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Lotta Siira

Clonality of *Streptococcus pneumoniae* in relation to antimicrobial resistance in Finland

RESEARCH

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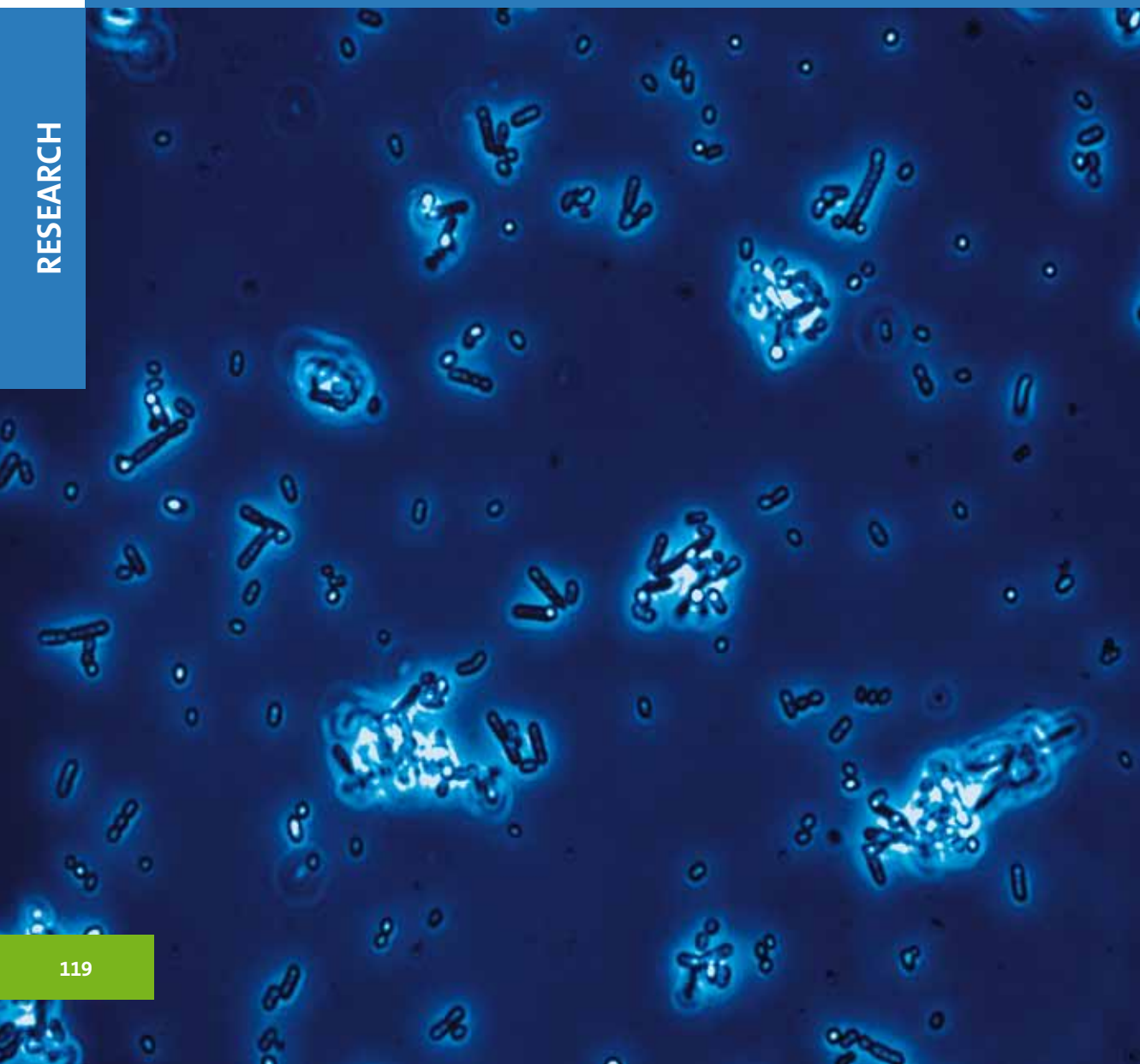

RESEARCH 119 • 2014

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Clonality of *Streptococcus pneumoniae* in relation to antimicrobial resistance in Finland

ACADEMIC DISSERTATION

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Bacteriology Unit, Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare

Research Program Unit, Immunobiology Research Program, University of Helsinki

Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki

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Cover photo: A positive Quellung reaction photographed by Lotta Siira

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Supervisor
Docent Anni Virolainen-Julkunen, MD, PhD
Department for Promotion of Welfare and Health
Ministry of Social Affairs and Health
Helsinki, Finland

Reviewers
Professor Ville Peltola, MD, PhD
Department of Pediatrics
University of Turku
Turku, Finland

Docent Mirja Puolakkainen, MD, PhD
Haartman Institute, Department of Virology
University of Helsinki
Helsinki, Finland

Opponent
Professor Birgitta Henriques-Normark, MD, PhD
Department of Microbiology, Tumor and Cell Biology
Karolinska Institutet
Stockholm, Sweden

"As he explained the apparatus and adjusted the lens, it seemed to him that by venturing beyond the visible world he had embarked on a voyage more perilous than he had known."

– *In the Reign of Harard IV* by Steven Millhauser

Abstract

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Streptococcus pneumoniae, or the pneumococcus, is a bacterium of the human normal flora that also causes non-invasive respiratory tract infections, and serious infections, such as pneumonia, septicaemia, and meningitis. Globally, the rise in pneumococcal antimicrobial resistance is a worrying trend. Over 90 pneumococcal capsules are recognised by serotyping; the most important serotypes are included in the available vaccines. The 10-valent conjugate vaccine has been part of the Finnish national vaccination programme since September 2010.

In this study, the serotype and genotype clonality of the invasive pneumococcal population in Finland was studied in relation to antimicrobial resistance. All invasive pneumococci isolated in Finland during 2002-2011 and a subset of non-invasive multidrug-resistant isolates from 2008 were serotyped and studied for antimicrobial susceptibility. The penicillin-resistant isolates were genotyped and pilus-encoding virulence genes were detected. A sequential multiplex PCR assay for serotyping was set up, tailored to the serotype distribution in Finland.

Serotype 14 was the predominant serotype, representing 17.5% of all invasive isolates. The proportion of isolates non-susceptible to penicillin and erythromycin was high, and it increased over the study period, reaching 22% and 26% for penicillin and erythromycin, respectively. The proportion of non-susceptible isolates was particularly high among isolates from children and in serotype 14. Among the genotyped isolates, international resistant clones dominated, but novel genotypes were also found, illustrating the continuous recombination of resistant pneumococci. The results of this study showed that in Finland a globally described multidrug-resistant serotype 19A clone appeared prior to large-scale vaccination. Conversely, in many countries, this clone has emerged following vaccination. Pilus-encoding gene carriage was frequent among the penicillin- and multidrug-resistant isolates. In the future, a vaccine targeting the pilus proteins would most likely be successful in controlling these clones. The new serotyping scheme is useful in surveillance, as knowledge of the serotype distribution of the invasive pneumococci is essential for vaccine development and monitoring of the vaccination programme.

Keywords: *Streptococcus pneumoniae*, clonality, serotyping, genotyping, antimicrobial susceptibility

Tiivistelmä

Lotta Siira, Clonality of *Streptococcus pneumoniae* in relation to antimicrobial resistance in Finland. [*Streptococcus pneumoniae* -bakteerin klonaalisuus suhteessa lääkeherkkyyteen Suomessa]. Terveyden ja hyvinvoinnin laitos. Tutkimus 119. 157 sivua. Helsinki, Finland 2014.
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Streptococcus pneumoniae, pneumokokki, on normaaliflooran bakteeri, joka voi aiheuttaa hengitystieinfektioita sekä vakavia tauteja, kuten keuhkokuumetta, verenmyrkytyksiä ja aivokalvontulehdusta. Pneumokokkien mikrobilääkeresistenssi on maailmanlaajuisesti ollut huolestuttavassa kasvussa. Pneumokokilla tunnetaan yli 90 eri polysakkaridikapselia, jotka määritetään serotyypittämällä. Käytössä olevat rokotteet kattavat tärkeimmät serotyypit. 10-valenttinen pneumokokki-konjugaattirokote on syyskuusta 2010 lähtien ollut osa kansallista rokotusohjelmaa.

Tässä tutkimuksessa tarkasteltiin invasiivisten pneumokokkien klonaalisuutta sekä serotyyppi- että genotyyppitasolla suhteessa mikrobilääkeherkkyyteen. Serotyyppi ja mikrobilääkeherkkyys määritettiin kaikille Suomessa vuosina 2002-2011 eristetyille invasiivisille kannoille sekä osalle vuonna 2008 eristetyille ei-invasiivisille moni-resistenteille pneumokokeille. Penisilliiniresistenteille kannoille määritettiin lisäksi genotyyppi ja pilusgeenien läsnäolo. Lisäksi pystytettiin Suomen pneumokokki-populaatioon räätälöity multiplex-PCR -pohjainen serotyypitysmenetelmä.

Serotyyppi 14 oli tärkein, se kattoi 17,5 % kaikista tutkimuksen invasiivisista pneumokokeista. Penisilliinille ja erytromysiinille herkkyydeltään alentuneiden kantojen osuudet olivat korkeat ja kasvoivat tutkimusjaksona kattamaan yli viidenneksen kannoista. Herkkyydeltään alentuneiden kantojen osuus oli erityisen korkea lapsilta eristettyjen kantojen keskuudessa sekä serotyypillä 14. Genotyypitetyt kannat olivat sukua maailmanlaajuisille resistenteille pneumokokki-klooneille. Myös uusia genotyyppejä havaittiin, mikä kuvastaa resistenttien kloonien jatkuvaa kehitystä. Havaittiin, että serotyypin 19A moniresistentti klooni on esiintynyt Suomessa jo ennen laajamittaisia rokotuksia, vaikka se on useassa maassa yleistynyt vasta rokotusten myötä. Valtaosa tutkituista resistenteistä kannoista kantoi pilusgeenejä. On todennäköistä, että mahdollinen pilusproteiineja sisältävä rokote kykenisi tulevaisuudessa torjumaan näitä kantoja. Pystytetyllä serotyypitys-menetelmällä voidaan selvittää invasiivisen pneumokokkipopulaation serotyyppi-jakauma. Sen tunteminen on ensiarvoisen tärkeää rokoteseurannassa ja rokote-kehittämistyössä.

Avainsanat: *Streptococcus pneumoniae*, klonaalisuus, serotyypitys, genotyypitys, mikrobilääkeherkkyys

Sammandrag

Lotta Siira, Clonality of *Streptococcus pneumoniae* in relation to antimicrobial resistance in Finland. Institutet för hälsa och välfärd. [*Streptococcus pneumoniae*-bakteriens klonalitet i relation till antibiotikaresistens i Finland]. Forskning 119. 157 sidor. Helsingfors, Finland 2014.
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Streptococcus pneumoniae, eller pneumokocken, är en bakterie som finns i människans normalflora men också orsakar allt från milda luftvägsinfektioner till svåra invasiva sjukdomar som lunginflammationer, sepsis och hjärnhinne-inflammationer. Globalt sett har antibiotikaresistensen bland pneumokocker ökat oroväckande under de senaste decennierna. Fler än 90 olika polysackaridkapslar har beskrivits; dessa bestäms genom serotypning. De viktigaste serotyperna finns med i de vaccin som utvecklats mot pneumokocksjukdomar. I september 2010 blev det 10-valenta konjugatvaccinet en del av det nationella vaccinationsprogrammet i Finland.

I den här undersökningen granskades de invasiva pneumokockernas klonalitet både på sero- och genotypnivå i förhållande till antibiotikaresistensen i Finland. Serotyper och antibiotikakänslighet bestämdes för alla invasiva pneumokocker som isolerades i Finland under åren 2002-2011 och för ett sampel av multiresistenta icke-invasiva pneumokocker från år 2008. De penicillinresistenta stammarna genotypades och deras gener för piluskodande virulensfaktorer utreddes. Ett nytt serotypnings-protokoll baserat på multiplex-PCR sattes också upp.

Bland serotyperna var serotyp 14 den viktigaste, den utgjorde 17,5% av alla invasiva stammar. Andelen isolat med nedsatt känslighet mot penicillin eller erytromycin var hög och ökade under forskningsperioden till 22 %, respektive 26 %, av stammarna. Andelen stammar med nedsatt antibiotikakänslighet var speciellt hög bland isolat från barn och inom serotyp 14. Internationella resistenta kloner dominerade bland de genotypade stammarna. Nya genotyper hittades också vilket beskriver de resistenta klonernas fortsatta utveckling. Resultaten visar också att den multiresistenta serotyp 19A-klon som på många håll i världen ökat markant efter vaccinering, hade fått fotfäste i Finland redan före storskalig vaccinering inletts. Majoriteten av de penicillin- och multiresistenta pneumokockerna bar på piluskodande gener, vilket tyder på att ett vaccin som innehåller pilusprotein i framtiden kunde begränsa dessa viktiga kloners framfart. Det nya serotypningsprotokollet möjliggör också i fortsättningen granskningen av pneumokockernas serotypfördelning för vaccin-uppföljning och vaccinutvecklingsbehov.

Nyckelord: *Streptococcus pneumoniae*, klonalitet, serotypning, genotypning, antibiotikaresistens

Contents

List of original publications

This thesis is based on the following original publications, which are referred to throughout the text by the Roman numerals given below (I-IV)

I Temporal trends of antimicrobial resistance and clonality of invasive *Streptococcus pneumoniae* isolates in Finland, 2002 to 2006. Siira L, Rantala M, Jalava J, Hakanen AJ, Huovinen P, Kaijalainen T, Lyytikäinen O, Virolainen A. Antimicrob Agents Chemother. 2009 May;53(5):2066-73.

II Clonality behind the increase of multidrug-resistance among non-invasive pneumococci in Southern Finland. Siira L, Jalava J, Tissari P, Vaara M, Kaijalainen T, Virolainen A. Eur J Clin Microbiol Infect Dis. 2012 May;31(5):867-71.

III From Quellung to multiplex PCR, and back when needed, in pneumococcal serotyping. Siira L, Kaijalainen T, Lambertsen L, Nahm MH, Toropainen M, Virolainen A. J Clin Microbiol. 2012 Aug;50(8):2727-31.

IV Antimicrobial resistance in relation to sero- and genotypes among invasive *Streptococcus pneumoniae* in Finland, 2007-2011. Siira L, Jalava J, Kaijalainen T, Ollgren J, Lyytikäinen O, Virolainen A. Microbial Drug Resistance. In press.

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Abbreviations

aroE	shikimate dehydrogenase gene
bp	base pair
CC	clonal complex
CDC	Centers for Disease Control and Prevention
CI	confidence interval
CIEP	counterimmunoelectrophoresis
CLSI	Clinical and Laboratory Standards Institute
cps	capsule polysaccharide synthesis locus
ddl	D-alanine-D-alanine ligase gene
DNA	deoxyribonucleic acid
eBURST	based upon related sequences
ECDC	the European Centre for Disease Prevention and Control
EQA	external quality assurance
erm	erythromycin ribosomal methylation gene
EUCAST	European Committee on Antimicrobial Susceptibility Testing
gdh	glucose-6-phosphate dehydrogenase gene
gki	glucose kinase gene
HUS	Hospital District of Helsinki and Uusimaa
I	intermediate
kb	kilo bases
LytA	autolysin, N-acetylmuromyl-L-alanine amidase
mAb	monoclonal antibody
Mb	mega bases
MDR	multidrug-resistance
mef	macrolide efflux gene
MIC	minimum inhibitory concentration
$MLKS_B$	lincosamide-ketolide-streptogramin B resistance phenotype
MLST	multi locus sequence typing
MLVA	multi locus variable number tandem repeat analysis
mPCR	multiplex PCR
NIDR	National Infectious Disease Register
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PEN	penicillin
PFGE	pulsed-field gel electrophoresis
PI-1	pilus islet 1
PI-2	pilus islet 2
PCV	pneumococcal conjugate vaccine
PCV7	7-valent pneumococcal conjugate vaccine

PCV10	10-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PMEN	Pneumococcal Molecular Epidemiology Network
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C
R	resistant
RR	risk ratio
recP	transketolase gene
S	susceptible
SLV	single locus variant
spi	signal peptidase I gene
SSI	Statens Serum Institute
ST	sequence type
THL	National Institute for Health and Welfare (Terveyden ja hyvinvoinnin laitos)
UAB	University of Alabama
WHO	World Health Organization
xpt	xanthine phosphoribosyl transferase gene
wzg	capsular regulatory gene, formerly named *cpsA*
wzy	capsular polymerase gene
wzy pathway	biosynthesis pathway for capsular polysaccharides

1 Introduction

Streptococcus pneumoniae, or the pneumococcus, is a commensal bacterium, which also causes infections of the upper respiratory tract and serious infections such as meningitis, septicaemia, and pneumonia. According to the Finnish National Infectious Disease Register, more than 700 invasive pneumococcal infections are diagnosed annually. The pneumococcus is asymptomatically carried in the nasopharynx especially by young children, with carriage rates decreasing with increasing age. Carriage is essential for disease to develop, and the strain causing disease tends to originate from the nasopharynx of the patient. The pneumococcus engages in both inter- and intraspecies competition in its natural habitat.

The pneumococcus is a diplococcus that is alpha-haemolytic when cultivated on blood agar. A capsule made of polysaccharides covers the bacterial cell and enables the bacterium to evade the immune system and is an important virulence factor. To date, more than 90 different capsular types, or serotypes, have been described. These differ in both immunogenicity and virulence and often, but not always, represent diverse genetic backgrounds. The most frequently occurring serotypes causing invasive disease are included in the available vaccines. The 10-valent pneumococcal conjugate vaccine is included in the Finnish national vaccination programme as of September 2010. The large-scale use of vaccines will assert serotype selection pressure that is likely to bring about changes both on the serotype and genotype level within the pneumococcal population. Over the last few decades, pneumococcal resistance to commonly used antimicrobial drugs has emerged. This is a worrying trend posing new treatment challenges.

The aim of this study was to examine the clonality of the invasive pneumococcal population, both on the serotype and the genotype level, and to set up a serotyping scheme tailored to study the invasive pneumococcal isolates in Finland. The study also examined a subset of multidrug-resistant non-invasive isolates that have increasingly been encountered. From a surveillance standpoint, these isolates are important, because changes in the non-invasive population are usually reflected in the invasive bacterial population in time. By combining virulence factor and clonal analysis the results of this study may be useful when future prevention strategies are considered and developed.

2 Review of the literature

2.1 The "sugar-coated microbe" and breakthroughs in the life sciences

Streptococcus pneumoniae, or the pneumococcus, is a Gram-positive, facultatively anaerobic catalase-negative round or lancet shaped diplococcus. It is fairly demanding to cultivate in the laboratory and generally thrives best in an atmosphere enriched with carbon dioxide. When cultivated on blood agar, it produces greenish alpha-haemolysis, as the hydrogen peroxide of the bacteria oxidises haemoglobin. The colonies are round and often dented in the middle, but the appearance depends on the capsular type, as some serotypes have a mucoid appearance. The bacterial cell is covered by a polysaccharide capsule, the structure of which determines the serotype of the bacterium. The capsule is an important virulence factor [3, 160].

The history of pneumococcal research mirrors the history of key findings and milestones in bacteriology and the life sciences. After the development of a light microscope with sufficiently high resolution to reveal bacteria that the naked eye could not detect, coccoid bacteria in pairs found in pulmonary tissues were reported in the literature in 1875 [111]. In 1881, the pneumococcus was described as a pathogen after it had been isolated independently by two researchers, George M. Sternberg and Louis Pasteur [11, 325]. Both found diplococcoid bacteria in the saliva of human carriers and both went on to inject the saliva into rabbits, thereby causing disease, and were able to recover the bacteria from the rabbit blood [325]. Since its discovery, the pneumococcus has been renamed several times. Its initial names *Microbe septicemique du salive* given by Pasteur and *Micrococcus pasteuri* by Sternberg, gave way to *Pneumococcus* a few years later, when its predisposition to cause respiratory tract disease became clear. In 1920, it went on to officially carry the name *Diplococcus pneumoniae*, given in an effort to describe both the shape and clinical manifestation of the bacterium. In 1974, the current name, *Streptococcus pneumoniae,* was adopted to indicate that in liquid media the bacteria grow in chains like other members of the *Streptococcus* genus. The pneumococcus was one of the first bacteria to be Gram-stained, a procedure developed by Hans Christian Gram in the 1880s and still relevant today in identifying clinically significant bacteria [325].

In 1923, the discovery that the pneumococcal capsule was comprised of polysaccharides, i.e. sugar, caused a stir. Until then, it had been widely accepted that only proteins were capable of acting as antigens and causing an immune response [296, 325]. Physician Oswald Avery, who was active in pneumococcal research for

several decades, affectionately called the pneumococcus the “sugar-coated microbe” [17]. Research into the pneumococcal capsule established it as a major virulence factor when it was discovered that it protects the bacterium from opsonisation and phagocytosis by the immune system. This research, in turn, developed into the demonstration of the microscopically visible Quellung reaction, in which the capsule swells upon addition of specific antiserum to pneumococci in liquid media (Figure 1) [12, 325]. This reaction is still commonly known by the German word for swelling, Quellung, or as the capsular reaction test, and is widely used for serotyping pneumococci. The number of known pneumococcal serotypes increased from two in 1910 to 85 some fifty years later [325]. Today, more than 90 different serotypes are known [23].

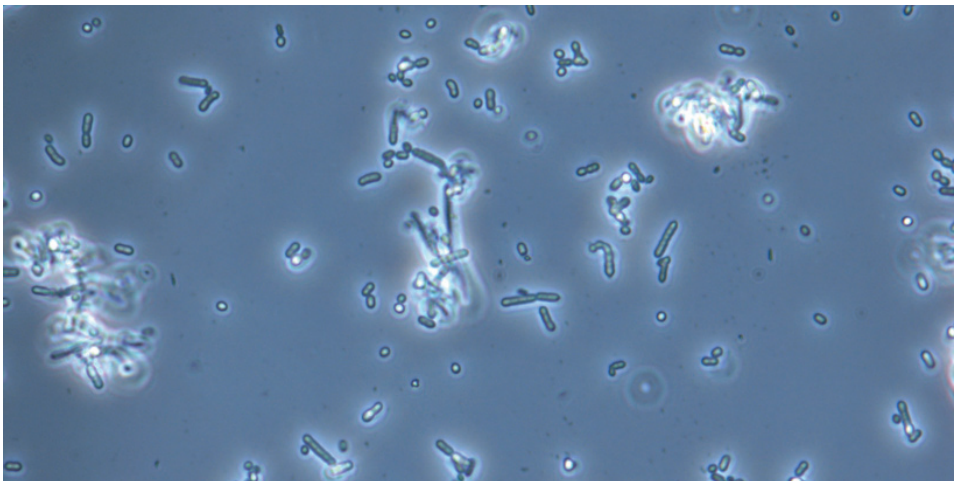

Figure 1. A positive Quellung reaction as seen in a phase contrast microscope.

Chemotherapy against pneumococcal infections, one of the first uses of specific antimicrobial agents as therapy for bacterial infections, took place as early as 1911. The agent in question was the quinine derivative ethylhydrocuperine, known as optochin, which specifically inhibits growth of pneumococci. Its therapeutic use was abandoned because of toxicity and rapidly developing resistance, but in the laboratory optochin remains a reliable tool for distinguishing pneumococci from other closely related species [33, 111]. The first successful use of penicillin in a clinical setting was against a pneumococcal conjunctivitis infection, establishing the clinical and therapeutic usefulness of the drug [325]. When penicillin was launched in the 1940s, it became the drug of choice and its use dramatically reduced mortality of serious pneumococcal infections [111].

The significance of breakthroughs made in pneumococcal research extends far beyond bacteriology. The best example of this is the discovery of deoxyribonucleic acid (DNA) as the hereditary molecule [14, 296]. This discovery made in 1944 built

upon the work from previous decades, when researchers had discovered that rather than being stable, an avirulent non-capsulated pneumococcus could become virulent when injected into a mouse simultaneously with heat-killed bacteria of a virulent capsulated strain [113]. This transformation of material that changed the phenotype of the strains in a so-called capsular switching event was shown to occur in liquid media as well as in laboratory animals. The chemical properties of the transforming molecule were consistent with those of DNA, although until then, it had been generally believed that protein was the genetic material. The finding was so ground-breaking that it took nearly a decade before it was widely accepted in the scientific community that DNA was the hereditary molecule and contained the genes of the organisms [201, 296].

2.2 Pneumococcal disease and carriage

In 2005, the World Health Organization (WHO) estimated that 1.6 million deaths annually were caused by the pneumococcus. Children under the age of 5 years account for 0.7–1 million of these deaths, and developing countries are most severely affected [333]. In 2008, it was estimated that 476,000 deaths in young children were caused by pneumococcal infections [332]. In developed countries, the pneumococcus is a common cause of community-acquired pneumonia in adults, sometimes accompanied by bacteraemia [29, 176]. In Europe, the age-standardised incidence of invasive pneumococcal disease was 5.12 per 100,000 population in 2010, according to the European Centre for Disease Prevention and Control (ECDC) [92]. However, the incidences show great variation between countries; in the years 2002 to 2005, they were 0.4 to 25.8 per 100,000 population [270]. In the Nordic countries, the age-standardised incidence per 100,000 population in the year 2010 was 14.82 in Sweden, 15.08 in Finland, 16.18 in Norway, and 17.26 in Denmark, respectively [92, 246]. In Finland, the incidences of both laboratory confirmed bloodstream infections as a whole, and invasive pneumococcal infections have increased over the past few decades [287, 300]. In 2012, 752 cases of invasive pneumococcal infections were registered in the National Infectious Disease Register (NIDR) [300]. In Finland, the incidence of pneumococcal bloodstream infections is slightly higher among males than females and the case fatality rate within a month is 10%. Nearly half of the deaths occur within two days of a positive blood culture sample [286]. The invasive pneumococcal disease incidence displays seasonal fluctuation, increasing in the winter months and correlating with the findings of influenza and other respiratory viruses [105, 298]. In Finland, a temporal association between invasive pneumococcal disease in young children and peaks in rhinovirus circulation during spring and autumn has been established [247]. Regional and ethnic differences have also been described in the incidence of invasive pneumococcal disease [189].

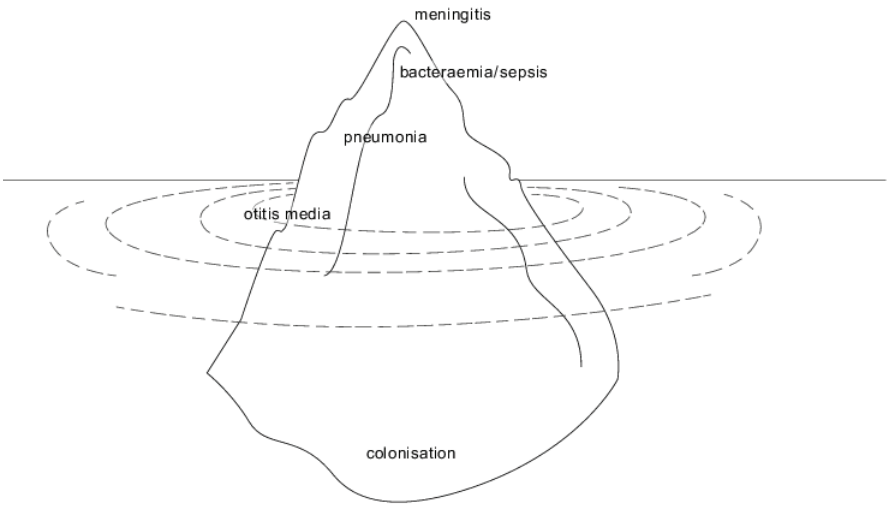

Figure 2. Pneumococcal colonisation and disease. Drawing by Hanna Siira.

The distribution of the clinical spectrum of pneumococcal colonisation and disease can be likened to an iceberg (Figure 2). Under the surface, the widest part is made up of colonisation; this is the most common situation, where the pneumococcus acts as a commensal. The visible part of the iceberg represents the clinical cases. The majority of these are non-invasive, relatively mild conditions, such as otitis media common in children, and other respiratory infections. Above are pneumonia and other serious conditions, and at the very top of the iceberg are bloodstream-infections, and finally, meningitis [160, 189].

Most pneumococcal disease cases are sporadic and transmission takes place from person to person though droplets or aerosols [134, 160]. Although rare and usually limited in size, invasive and non-invasive outbreaks have been described in confined settings such as nursing homes, hospital wards, day care centres, military camps, and homeless shelters [27, 104, 114, 143, 264, 309]. In the so-called meningitis belt in Africa, invasive outbreaks associated with serotype 1 have occurred [178, 225], and serotype 15F strains has been found to cause outbreaks in rural communities in Alaska [347].

Host risk factors for pneumococcal carriage and disease include alcohol abuse, smoking or exposure to tobacco smoke, asthma, and acute upper respiratory infections [189]. Day care or school attendance and more than four co-habitants are risk factors for invasive pneumococcal disease in children [52, 53]. Human immunodeficiency virus infections and the acquired immunodeficiency syndrome are predisposing conditions to pneumococcal disease, as is any other

immunocompromising condition and old age [189, 322]. Young children are also susceptible to pneumococcal disease mainly because their immune systems have yet to fully develop and therefore are not able to quickly eradicate polysaccharide covered pneumococci [332]. The factors of and interplay between both the bacterium and the human host determine whether the pneumococcus is able to cause disease, as well as the severity and clinical manifestation of the disease. Disease is a relatively rare event compared to carriage. Upon acquisition, a strain may be carried for weeks or even months in the nasopharynx, where both innate and adaptive immune responses are involved in limiting the pneumococci [135, 160]. In carriage, the pneumococcus adheres to the resting epithelial lining of the nasopharynx. For disease to develop, the bacterium must spread from the nasopharynx, either locally when causing sinusitis or otitis media, by aspiration into the alveoli when causing pneumonia, or by invading the bloodstream when causing septicaemia. In meningitis, the brain-blood barrier is breached and the pneumococci reach the cerebrospinal fluid [31].

The carriage rate is high during the first two years of life and declines thereafter. In healthy 18-month-old children in a study conducted in the Netherlands it was 12% [30], while it was as high as 55.5% in children below 24 months in a study from the UK [245], 49.3% in 4 to 12-month-old children in Bangladesh [109], and 9-43%, increasing with age, in 2 to 24-month-old healthy children in a Finnish study [297]. Age-related decline in pneumococcal carriage is caused by the maturation of the immune system and occurs parallel with simultaneous increase in *Staphylococcus aureus* carriage [32]. The nasopharynx of children can be considered the natural habitat of the bacterium and the reservoir from where it may be transmitted. Adults in families with young children are often more likely to carry the bacterium than their peers living in families without children. The adult carriage rate varies in different populations and in different settings but is often below 10% [134].

The pneumococcus has sporadically been encountered in pets, as well as zoo and laboratory animals [314], but humans are its main host. Nasopharyngeal colonisation is a dynamic process, in which carried species and serotypes are in flux and vary by age, season, geographical area, genetic background, and is further influenced by socioeconomic factors. Interventions, such as the use of antimicrobial agents and vaccines, also have an impact on the dynamic [30-32]. The pneumococci found in carriage and non-invasive samples are more diverse than the strains most commonly isolated from invasive samples [121, 175]. The pneumococcus has a complex relationship with the estimated 700 other bacterial species that share this niche [160]. The resident bacterial flora, which includes alpha-haemolytic species, inhibits the colonisation of invading species, such as pneumococci, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Furthermore, several of the pathogens have competitive relationships with the other species [31]. The pneumococcus can interfere with the

growth of *S. aureus, M. catarrhalis, H. influenzae,* and the meningococcus *Neisseria meningitidis* [31, 32, 248]. On the other hand, meningococcal presence *in vitro* increases the growth of pneumococci [31].

2.3 Pneumococcal virulence factors

The pneumococcus is able to adapt its gene expression in a site specific manner and its different virulence factors play varyingly important roles depending on the strain, as well as on the type and stage of disease, as [160, 239]. The most important virulence factor of the pneumococcus is the polysaccharide capsule [192], which is discussed in detail in the next section. However, the bacterium also has other virulence factors that facilitate colonisation and survival in the host; some of the most central are discussed below.

The exotoxin pneumolysin is expressed by nearly all invasive pneumococcal isolates, and several different variants of the molecule are known [160]. Pneumolysin is cytolytic at high concentrations, when the soluble proteins oligomerise in the cholesterol-containing membranes of the target cells to form large round pores consisting of more than 40 subunits [305]. At lower concentrations, pneumolysin is cytotoxic and interferes with the immune defence by influencing ciliary beating, complement activation, and induction of intracellular oxygen radicals [160, 198]. The role of pneumolysin in pneumococcal virulence in pneumonia is well established. It also seems to be important in the survival and spread of bacterial from the lungs to the bloodstream and for the clinical manifestation of bacteraemic infections [161, 238]. Its role in meningitis remains controversial [160].

Several protein structures that influence virulence are located on the surface of the pneumococcal cell. The most recently discovered are pili, hair-like adhesive structures that protrude from the bacterial surface [73, 230]. The pneumococcal pili are encoded by two pilus islets, PI-1 and PI-2, on the bacterial chromosome. These were revealed by whole-genome sequencing. Clinical and carriage isolates may carry none, one, or both of the islets [1]. The expression of PI-1 has been shown to mediate adhesion to host cells and provide a competitive advantage in an animal model of respiratory tract colonisation [18, 230]. Initially, this pathogenicity islet was named *rlrA* islet, after its positive regulator gene. Pneumococcal PI-1 carriage is associated with antimicrobial non-susceptibility [2]. The presence of PI-1 is a clonal property, with a stronger association with the genotype than the serotype [2, 223]. Expression of PI-2 also mediates adherence to host cells [16]. In contrast to PI-1, PI-2 is associated with antimicrobial susceptibility, although dual carriage of PI-1 and PI-2 is associated with antimicrobial resistance [1, 343].

On its surface, the pneumococcus also carries several choline-binding proteins that interact with host structures and influence virulence. The proteins are anchored by their homologous C-terminal parts to the pneumococcal cell wall phosporylcholine and vary in their protruding parts [118]. One of the choline-binding proteins is the pneumococcal surface protein A (PspA), a variable molecule that prevents complement mediated killing of the bacteria [141, 160]. Another is the pneumococcal surface protein C (PspC), also known as choline-binding protein A, which helps the bacteria adhere to epithelial cells and promotes nasopharyngeal colonisation [160].

After reaching the stationary phase in the growth curve, the pneumococcus undergoes characteristic autolysis by degrading its cell wall and thereby inducing its own death. This trait seems to add to virulence and protect intact bacteria from clearing by the immune system [196, 198, 336]. The major autolysis inducing enzyme autolysin (LytA), or N-acetylmuromyl-L-alanine amidase, severs bonds in the peptidoglycan cell wall [160]. Several theories of why LytA influences virulence have been suggested: its induction releases other virulence factors or toxins for instance pneumolysin and the cell wall components, and its induction may hinder phagocyte activities [160, 198]. Together with pneumolysin, LytA is essential for the survival of the pneumococcus in the bloodstream [238].

Pneumococcal surface adhesin (PsaA) is a lipoprotein located at the bacterial cell wall that appears to be involved in providing resistance to oxidative stress [160]. Teichoic acid and peptidoglycan, both major cell wall components, induce inflammation [307], while other pneumococcal virulence factors include LPXTG-anchored proteins such as neuraminidases and pneumococcal histidine triad proteins [118].

2.4 The pneumococcal capsule

The capsule is the most important virulence factor for invasive pneumococcal disease [160, 192]. The capsule inhibits complement and protects the bacterial cell from neutrophil-mediated killing, while the protection increases with the degree of encapsulation [160, 329]. Strains with a thick capsule are more virulent and prone to cause invasive disease, while strains with a thinner capsule are more often found in asymptomatic carriage [340].

More than 90 different pneumococcal serotypes have been described to date, but a smaller number is responsible for most invasive disease. Globally, more than 80% of invasive disease is caused by around 20 serotypes [158, 333]. The serotype distribution of the invasive pneumococci varies depending on time, place, and age-

group [126, 127, 284]. Fluctuations in the serotype frequencies or proportions may take place over time even without selection pressure asserted by interventions [99, 122]. In children under 5 years of age, serotype 14 is the most common cause of invasive pneumococcal disease in all regions. Often it is an important cause of invasive disease in other age-groups as well [126, 158]. The serotypes differ in genetic, immunological, biochemical, and epidemiological properties [23, 277]. Indeed, they show such variation in their properties, epidemiology, and invasive disease outcomes, that from an epidemiologic point of view, it has been suggested that each of them should be considered a separate pathogen [127, 328]. The risk factors, disease focus, and clinical presentation are partly associated with serotype [53, 284, 315, 347], and carriage efficiency also depends on the capsule [125].

Certain serotypes or serogroups display high invasive disease potential, i.e. they exhibit a high propensity for causing invasive disease relative to the exposure through carriage, while others show low invasive disease potential, i.e. they are common in carriage but proportionately rarer in invasive disease episodes. These serotypes or serogroups are summarised in Table 1. As illustrated, the invasive disease potential of some serotypes, such as serotype 3, exhibit differences between studies. Interestingly, high invasive disease potential has not been linked to high mortality [285], but serotypes 3, 6A, 6B, 9N, 11A, 19F, and 31 are associated with increased risk of death [285, 315, 328]. Underlying conditions allow serotypes with otherwise low invasive disease potential to act as opportunistic pathogens and cause invasive disease [285]. Just as the capsule affects the pathogenesis of a pneumococcal strain, non-encapsulated isolates also display particular characteristics and appear to have a propensity to cause conjunctivitis [119].

Table 1. Serotypes or serogroups with high or low invasive disease potential, respectively, as identified in three studies.

Serotypes/groups with high invasive disease potential	Serotypes/groups with low invasive disease potential	Reference
1, 5, and 7	3, 6A, and 15	[38]
6B, 14, 18C, and 19A	6A and 11A	[121]
3, 7F, 18C, 19A, 22F, and 33F	6C, 11A, 15A, 15B/C, 19F, 23A, 35B, and 35F	[339]

To date, 97 different pneumococcal serotypes or capsular types belonging to 46 serogroups have been published [23, 34, 42, 43, 132, 164, 237, 244, 345]. In addition to these serotypes, the DNA sequence of a novel serogroup 33 subtype, proposed to be named 33E, is available in the nucleotide sequence database (accession numbers EU071709 and EU071709 [302]). Currently, not all of the most recently described serotypes are distinguishable by conventional antisera [42, 164,

275]. As both genetic and immunologic methods for studying pneumococcal serotypes evolve, it is likely that new serotypes or further structural subtypes of previously described serotypes are discovered [85, 203, 275].

2.4.1 Capsular genes, structure, and production

The capsule is the outermost layer of the pneumococcal cell. Its thickness is 200 to 400 nm and varies considerably depending on the serotype [288]. The structure of the capsular polysaccharides varies depending on the serotype and may be linear or branched. Branching is determined by the enzymes catalysing the polymerisation of the polysaccharide [340]. The structure of the capsular polysaccharides also affects the prevalence of the serotype in carriage [329].

All but two of the pneumococcal capsular polysaccharides are synthesised and transferred to the cell surface through the so-called *wzy* pathway. This pathway is also used in some Gram-negative bacteria and in nearly all other Gram-positive bacteria for capsular synthesis [340]. The proteins required for the *wzy* pathway are encoded by genes located in the capsule polysaccharide synthesis locus *cps*, which can be found between *dexB* and *aliA* on the pneumococcal chromosome (Figure 3). The locus varies in size from 10,337 bp for serotype 3, to 30,298 bp for serotype 38, depending on the specific genes required for the synthesis of each serotype [23]. Within the locus, the genes were originally named *cps* to which a letter was added for each individual gene in the sequence, i.e. *cpsA, cpsB, cpsC, cpsD* etc. More recently, the *cps* genes have been re-named by function and orthology. This allows homologous genes in different serotypes and across different species to carry the same name regardless of their relative location within *cps*. The aforementioned four genes are named *wzg, wzh, wzd*, and *wze* in most serotypes (Figure 3) [23, 156, 340]. At the 5' region, the *cps* locus contains the four conserved genes, which are involved in the modulation of the capsular synthesis [156, 340]. In one study, expression of the regulatory gene *wzg* (*cpsA*) correlated inversely with the thickness of the capsule [125]. Located downstream from the conserved genes are the serotype specific genes that encode enzymes for carrying out the polymer-specific tasks. In most serotypes, these enzymes consist of glycosyltransferases, polymerases, flippases, transferases, nucleotide dephospho-sugar synthases, and modification enzymes, such as *O*-acetylases [340].

Sugars needed for polysaccharide assembly are synthesised in the cytoplasm of the bacterial cell by housekeeping genes or *cps*-encoded genes, depending on the serotype [340]. The hypothetical biosynthesis of the capsule has been described as follows. The first transferase, WchA, links the initial sugar to a membrane-associated lipid carrier on the inside of the cytoplasmic membrane. Further glycosyl transferases sequentially link sugars to form a repeat unit, which upon completion is

transported across the cytoplasmic membrane by the Wzx flippase. The Wzy polymerase attaches individual repeat units to form lipid-linked capsular polysaccharides. The Wzd/Wze complex located in the cytoplasmic membrane and the inner wall zone translocates the mature polysaccharides and may also be responsible for their attachment to the peptidoglycan surface (Figure 3) [23].

The only two capsular types, serotypes 3 and 37, which are not synthesised by the *wzy* pathway, are produced using the synthase dependent pathway [186, 340]. This pathway involves fewer genes than the *wzy* pathway and the capsular polysaccharides are simpler. The *cps* of the serotype 3 capsule is located between *dexB* and *aliA*, just as the *wzy* pathway genes, although the conserved genes are non-functional [10]. In serotype 37, this locus is occupied by a defect 33F-like sequence and the single *tts* gene required for capsular synthesis is located elsewhere on the chromosome [186, 340].

Pneumococci carrying and expressing more than the genes for one capsule following transformation experiments have been described. However, not only are these kinds of isolates extremely rare, but they are also unstable [13, 26]. The *cps* locus displays heterogeneity and in some cases the difference between serotypes can arise from a difference in a single nucleotide position. For example, in serotypes 6A and 6B a single nucleotide polymorphism in the rhamnosyl-transferase gene *wciP* changes the amino acid in position 195 from serine in 6A to asparagine in 6B. This accounts for the difference between the two capsules [200]. That changes in the *cps* locus affect the capsular production is also illustrated in the two types of spontaneous phase variation exhibited by the pneumococcus, both of which at least partly involve the capsule. Firstly, the colony morphology can switch from a thickly encapsulated opaque to a thinly encapsulated transparent form. This may have implications for colonisation, as the transparent form exhibits higher colonisation rates in animal models, while a thicker capsule adds to virulence. The exact mechanism of switching from the transparent to opaque form is not fully known [321]. Secondly, the other type of phase variation, which involves switching on and off the capsular production in a single strain, has been observed in the laboratory for serotypes 3, 8, and 37 [323, 324]. Furthermore, serotypes 15B and 15C can interchange from one to the other at a frequency of up to 1 in 250 by the mechanism of slipped-strand mispairing in a tandem repeat sequence within one of the *cps* genes [316].

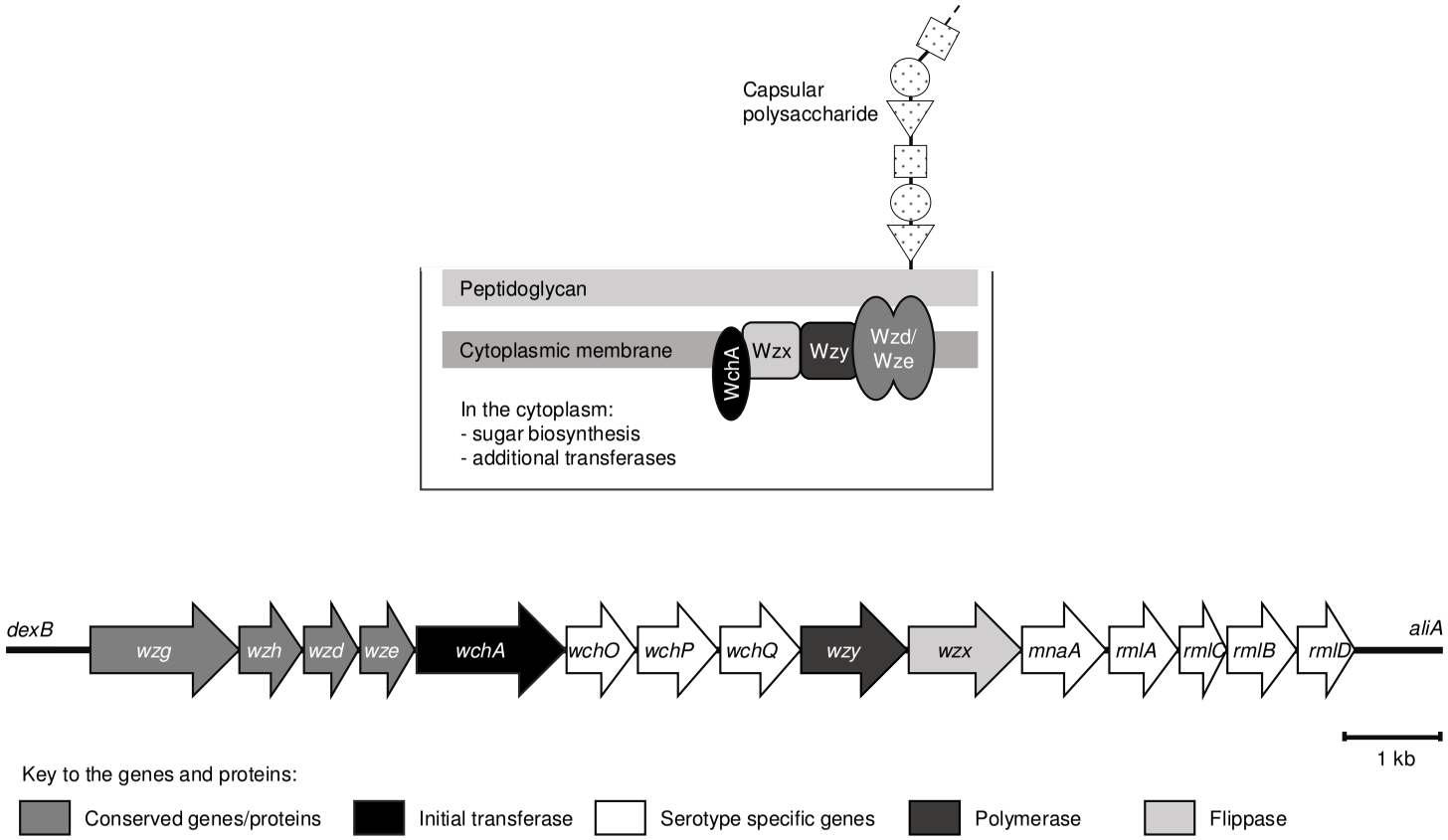

Figure 3. The upper panel shows the proposed positions of some of the key enzymes in the Wzy pathway mediated pneumococcal capsular synthesis, adapted from Bentley *et al.* 2006 and Yother, 2011 [23, 340]. Below the genes of the serotype 19F *cps* locus are depicted, adapted from Bentley *et al.* 2006 and Morona *et al.* 1999 [23, 220]. The key to the colours of the genes and proteins is shown at the bottom of the figure.

Regulation of capsule expression probably takes place both at the transcription and post-transcription level. The *cps* affects the growth curve of the strain in the laboratory, as the capsules differ in metabolic burden. The growth pattern is transferable from one strain to another through capsule switch mutations. It further correlates with carriage prevalence, so that the serotypes with a more pronounced lag phase due to the burden of capsule production are less common in carriage and require more nutrient-rich growth media [125]. It has been proposed that high serotype prevalence in carriage is associated with metabolically less costly capsules that contain few carbons per repeat unit. These serotypes include 3, 6A, 6B, 14, 19F and 19A. As these serotypes are able to be more heavily capsulated without being metabolically burdensome for the bacterium, they are well protected from the immune system also when causing disease [329].

Given that the capsule is an important virulence factor, non-encapsulated isolates are only rarely encountered in clinical specimens [160, 278]. Studies of non-typeable isolates have revealed that several of the examined invasive isolates actually carried *cps* genes, although capsular detection by phenotypic means had been unsuccessful. It is possible that capsule production was downregulated *in vitro* [242, 278]. Non-encapsulated pneumococci can also be grouped into clades, some of which are as successful at colonising mice as capsulated isolates [242]. Many of the restudied non-typeable carriage and non-invasive isolates did not carry any *cps* genes, or only carried disrupted genes that would not allow for capsular production [197, 278].

2.4.2 Serotype nomenclature

In 1974, the Danish serotyping nomenclature was adopted internationally [122, 131]. Serotypes are known either solely by a number, e.g. serotype 14, or if serologically similar serotypes are known, these are gathered together into serogroups named by numbers, e.g. serogroup 7. For each serotype within a serogroup, a letter suffix is added, e.g. serotypes 7F, 7A, 7B, and 7C. In the majority of the serogroups, the first serotype to be described was assigned the letter F and any subsequent serotypes were named alphabetically starting with A [131]. The serotype nomenclature is based on the immunological properties of the capsule. However, the genetic study of *cps* revealed that sequence similarity is not necessarily greater within a serogroup than outside it. For example, serotypes 7B and 7C share a greater sequence similarity with serotype 40 than with serotypes 7F and 7A within the same serogroup [23].

2.5 Antimicrobial resistance

The advent of the era of antibiotics reduced the mortality of serious pneumococcal infections and penicillin continues to be the primary antimicrobial drug for treating pneumococcal infections. [111]. For a long time, the pneumococcus was considered

to be universally susceptible to penicillin [117], although already in 1943, researchers had demonstrated that resistance to penicillin could be induced in the laboratory. Some 25 years after penicillin had become available and used in therapy, the first clinical pneumococcal strain that was non-susceptible to the drug was described in 1967 in Australia and soon after elsewhere [5, 60, 183]. Initially, the elevated minimum inhibitory concentrations (MICs) of the non-susceptible isolates were fairly low at 0.6 mg/L. The issue did not receive much attention until a decade later, when outbreaks involving invasive cases caused by pneumococci with higher level MICs (2-8 mg/L) were described in South Africa. The epidemic strain also exhibited resistance to chloramphenicol [5]. Following this observation, resistance to several other classes of antimicrobials such as macrolides, clindamycin, and tetracycline was described [5]. Since the 1980s and 1990s, non-susceptible or resistant pneumococci have been increasingly encountered in both invasive and non-invasive samples all over the world [117]. Non-susceptibility to antimicrobials is most frequently observed in pneumococcal clones common among children and in carriage [137, 190, 291]. The reason is that carriage in children tends to be longer than in adults and their antimicrobial consumption can be frequent [291].

The clinical importance of pneumococcal non-susceptibility is a topic that is still much debated, but there seems to be agreement that at least resistance with high MIC is likely to have clinical significance [190]. Critics say factors independent of antimicrobial susceptibility may cloud the issue. Clinical failures may reflect circumstances other than the antimicrobial susceptibility of the pneumococcus. These include underlying disease, comorbidities, or old age of the patient, while other factors may be properties of the infecting strain, such as the serotype [190]. Some studies suggest penicillin-susceptibility has no major impact on the fatality rate of invasive pneumococcal disease episodes, but it is also important to note that certain serotypes with high invasive disease potential, such as serotype 1 and 7F, are only rarely observed to be non-susceptible to penicillin [285]. In a setting with low prevalence of resistance, antimicrobial drugs lower the risk of pneumococcal carriage [245]. However, a Canadian cohort study concluded that to predict the appropriate antimicrobial therapy for invasive pneumococcal disease, the physician should be aware of any antimicrobial use in the previous three months, as this is the most important risk factor for acquisition of a resistant strain [317]. Selection pressure brought on by antimicrobials is a risk factor for the acquisition of a non-susceptible stain [6, 190].

Hand in hand with the clinical implications of resistance goes the interpretation of the susceptibility test results in the clinical and reference laboratories. The Clinical and Laboratory Standards Institute (CLSI), USA, issues susceptibility breakpoints for various pathogens and classes of antimicrobial agents. The breakpoints for bacteria issued by the European Committee on Antimicrobial Susceptibility Testing

(EUCAST) have been available since 2010, and differ in some parts from the CLSI breakpoints [93, 194]. Microbes are categorised by the use of breakpoints into susceptible (S), intermediate (I), and resistant (R) isolates. The intermediately and resistant isolates are collectively referred to as non-susceptible. In 2008, the CLSI breakpoints for pneumococcal penicillin-susceptibility were revised, taking into consideration clinical, pharmacokinetic, and microbiological aspects. Previously, one set of breakpoints had covered all types of cases, while the new breakpoints differentiate between meningitis and other cases [330]. By applying the new breakpoints, a larger proportion of the non-meningitis isolates are shifted to the penicillin-susceptible category, while a larger proportion of the meningitis isolates are moved to the resistant category [48].

2.5.1 Modes of antimicrobial action and resistance mechanisms

Just as the mode of antimicrobial action varies, the pneumococcal resistance mechanism also varies depending on the antimicrobial agent.

Penicillin, cephalosporins, and other β-lactam antibiotics disrupt the integrity of the bacterial cell by interfering with a group of enzymes known as penicillin-binding proteins (PBPs) involved in the cell wall synthesis, eventually leading to bacterial lysis [344]. Cephalosporins have been improved upon in stages, so that each new generation of drugs has broader spectrum of antimicrobial activity than the previous one. Resistance to β-lactams in pneumococci is conferred by PBPs with lower affinity for penicillin. These PBPs develop through mosaic changes in the PBP-encoding genes. While penicillin mainly reacts with PBP2b, the most important target for cephalosporins is PBP2x [95]. The changes affecting these proteins are acquired through homologous recombination of genes from other streptococci, whether other pneumococcal strains or other related species [60]. Four of the six pneumococcal PBPs are strongly implicated in resistance. Certain amino acid changes in PBP2b and PBP2x render the bacterium resistant, and additional changes in PBP1a are essential for high resistance [112, 289, 344]. Changes in PBP2a may also increase resistance further [60], and some of the other PBPs may occasionally be involved in resistance [117]. High cephalosporin-resistance, especially, cannot be explained solely by alterations in the PBPs [95]. It appears that mosaic changes in *murMN* operon, which encodes enzymes for branching peptidoglycan muropeptides, is vital for β-lactam resistance and that other mutations may also play important parts [60, 95, 96]. Pneumococcal penicillin-resistance is a complicated multifactorial process, and the variation of mosaic *pbp* alleles among resistant isolates is very high [117].

Macrolides were first discovered in the 1950s. Globally, pneumococcal macrolide-resistance appeared in tandem with penicillin-resistance. Erythromycin and other

macrolides impede bacterial growth by binding to the 23S ribosomal RNA and inhibiting protein synthesis. Pneumococcal macrolide-resistance is usually mediated by either target site modification or active drug efflux, or both [60]. Target site modification is most commonly carried out by post-transcriptional methylation of the adenine in position 2058 of the pneumococcal 50S ribosomal subunit, which reduces attachment of the drug to its target site [84]. Methylation is performed by an enzyme usually encoded by the gene *erm*(B) or in some cases by *erm*(A) [60]. Expression of the transposon carried *erm*(B) results in resistance also against lincosamides, ketolides, and streptogramin B that all share a similar molecular target. This phenotype is named the "$MLKS_B$ phenotype" after the collective abbreviation of the antimicrobial agents in question [84, 318]. Active efflux of the drug from the bacterial cell is mediated by pumps encoded by the genes *mef*(A) or *mef*(E). This results in the so called "M phenotype" which provides resistance only against macrolides and displaying lower MICs than the $MLKS_B$ phenotype [84]. Dual phenotype isolates, i.e. isolates that carry both the *erm* and *mef* resistance determinants, exhibit a very high level of macrolide-resistance and are found especially among multidrug-resistant isolates [60]. In addition to target site modification and active efflux, pneumococci may carry ribosomal mutations that provide resistance by preventing the drug from binding to its target site. Such changes are similar to *erm*-mediated target site modification in that they often provide resistance to more than one group of antimicrobial agents [84].

Tetracycline antimicrobials are bacteriostatic drugs. Their main mode of action is blocking the bacterial protein synthesis by binding to the 70S ribosome. Recent archaeological finds suggests that tetracycline exposure may have been prolonged among the members of a Nubian tribe during Late Antiquity more than 1,600 years ago, but in the modern era, tetracycline was first taken into clinical use in the late 1940s. For pneumococcal infections it is generally prescribed if other antimicrobials, such as penicillin, are contraindicated [231]. Tetracycline resistance is common among the bacteria found in the oral cavity, and can spread between oral and non-oral bacteria [226]. Pneumococcal tetracycline resistance is mediated by ribosome protection proteins encoded by *tetM* or occasionally by *tetO* [253, 318]. The *tetM* resistance gene may be located on the same transposon as *erm*(B), in which case the acquisition of macrolide and tetracycline resistance often occurs simultaneously [318].

Fluoroquinolones were originally formulated to treat Gram-negative bacterial infections. Their development against Gram-positive bacteria was prompted by the growing resistance of pneumococci against β-lactam antibiotics. Fluoroquinolones, such as levofloxacin, disrupt cell division by attaching to gyrase and topoisomerase enzymes that are essential for the supercoiling of DNA and for chromosome segregation [190]. Fluoroquinolone resistance is mediated by single-nucleotide polymorphisms, mutations in the genes encoding these enzymes leading to alterations in the

fluoroquinolone binding sites [67, 190]. In contrast to resistance against β-lactams, fluoroquinolone resistance is often the result of gradual accumulation of sporadic mutations in the quinolone resistance determinant region [60].

2.5.2 Selection pressure and fitness cost of resistance

Resistance to antimicrobial agents carries a fitness cost to the bacterium, and is more common in carriage isolates especially from children than in invasive isolates [291]. To offset the fitness cost of resistance, pneumococci may employ a number of strategies [60]. In one strategy, compensatory mutations, i.e. additional mutations in genes not directly involved in resistance, allow the maintenance of the resistance mechanism also in environments void of antimicrobial selection pressure by fully compensating for their fitness cost. Heteroresistance, on the other hand, is the presence of bacterial subpopulations that are capable of growth in higher antimicrobial concentrations than the bacterial population as a whole. It has been suggested that heteroresistance may allow pneumococci to test out resistance without the full fitness cost of a resistance mechanism [60, 219].

The development of resistance has been linked to antimicrobial drug consumption [254]. Subinhibitory concentrations of antimicrobials, which may occur in the body during or following treatment, induce an increase in pneumococcal mutation frequency, the so called hypermutation phenotype, which may give rise to resistance [60]. In studies conducted in Finland, high macrolide, trimethoprim-sulfametoxazole, and azithromycin consumption has been shown to increase pneumococcal resistance against these antimicrobials. The studies also linked β-lactam and cephalosporin consumption to low-level penicillin-non-susceptibility [25, 254]. A large European study also demonstrated this correlation both for the consumption levels of individual antimicrobial classes and for total antimicrobial consumption [268].

Non-invasive isolates usually display non-susceptibility to more antimicrobial agents than invasive isolates [137], possibly because the non-invasive isolates are exposed more often and to a greater number of antimicrobial agents than invasive isolates. However, as the same isolates may be involved in both invasive and non-invasive disease, it has been suggested that optimal surveillance of antimicrobial resistance should take into account also a subset of non-invasive isolates and the drugs used for treatment of infections [175].

2.6 Resistance and clonality

Pneumococcal resistance is clonal when certain genetic lineages or serotypes account for a large proportion of the non-susceptible isolates. Pneumococcal non-susceptibility and resistance to penicillin and macrolides is mainly clonal, while the

clonality of resistance to fluoroquinolones is controversial, but unlikely to be clonal [60, 190]. In response to the observation of clonal antimicrobial resistance, the Pneumococcal Molecular Epidemiology Network (PMEN) was founded in 1997. The aim of the network was to standardise clone nomenclature and to curate a collection of well-characterised and globally disseminated clones [204, 261, 261]. To date, the collection includes forty-three clones with a wide geographic spread. The clones have been characterised by serotyping, antimicrobial susceptibility testing, multi locus sequence typing (MLST), and sometimes additional methods, such as *pbp* gene pattern analysis. Upon acceptance into the collection, PMEN assigns numbers to the clones and names them after their original country of isolation. In recent publications, the clones are sometimes referred to as PMEN and their clone number, e.g. PMEN1, rather than Spain^{23F}-1 or Spain^{23F}ST81. In this text, the clones will be referred to by a combination of the two, i.e. PMEN1 Spain^{23F}ST81. This includes the PMEN clone number (PMEN1), original country of isolation (Spain), serotype (23F), and sequence type (ST81) as defined by MLST. More recently, the PMEN collection was expanded to include pneumococcal clones that are susceptible to antimicrobial agents, but clinically important and globally disseminated [261].

While all the PMEN clones have spread to at least two continents, their importance may vary between countries. By the late 1990s, 40% of the penicillin-non-susceptible isolates studied in the USA were related to PMEN1 Spain^{23F}ST81 [60, 61], while PMEN2 Spain^{6B}ST90 was responsible for the increase in penicillin-non-susceptibility in Iceland in the early 1990s [172], and nearly half of the penicillin-non-susceptible isolates in Poland in 2003-2005 and in Sweden in 2003 were related to PMEN3 Spain^{9V}ST156 [273, 284].

Serotype variants of the PMEN clones have been widely described. PMEN1 Spain^{23F}ST81 has been found to express at least eight different capsules in addition to the original serotype 23F [67, 139, 227]. PMEN3 Spain^{9V}ST156, originally described as serotype 9V, was later widely found also displaying serotypes 14, 19F, 19A, 11A, or 15C [57, 227]. The genetic lineage around the PMEN1 Spain^{23F}ST81 clone looks fairly conserved when compared to other genetic pneumococcal lineages (Figure 4), displaying characteristics more often associated with young clones [293]. However, there are significant differences between isolates within the clone and even with the same serotype, regarding systemic virulence and moribundity in test animals [139, 147]. The ancestral strain of PMEN1 Spain^{23F}ST81 is likely to be one of the first penicillin-non-susceptible pneumococci isolated in 1967 in Australia [337], but mutations providing resistance to non-β-lactam antimicrobials have been acquired multiple times within the lineage either through horizontal gene transfer or spontaneous mutations [67]. The DNA of PMEN1 Spain^{23F}ST81 is also highly dispersed within the pneumococcal population and the clone has acted as a donor

especially for antimicrobial resistance determinants. For example, the PMEN1 and PMEN3 reference strains share identical *pbp2x*, *pbp1a* and *pbp2b* alleles that provide penicillin-resistance [337]. Genotypic variants differing from the PMEN clones in one or a few analysed genomic loci are also common. This illustrates the diversification of successful clones and the plasticity of the genome, although the number of variants and subgroup founders described vary markedly between genetic lineages (Figure 4) [227].

Non-encapsulated pneumococci are often non-susceptible to penicillin and can play a role in the development of resistance of capsulated isolates and clones. The rate of transformation of non-encapsulated isolates is higher compared to capsulated isolates, and by harbouring resistance genes, they are able to act as a vector for resistance [197]. The development from low to higher penicillin MIC by the transfer of resistance genes from non-encapsulated isolates has been demonstrated in the PMEN21 Portugal^{19F}ST177 clone in Switzerland [128].

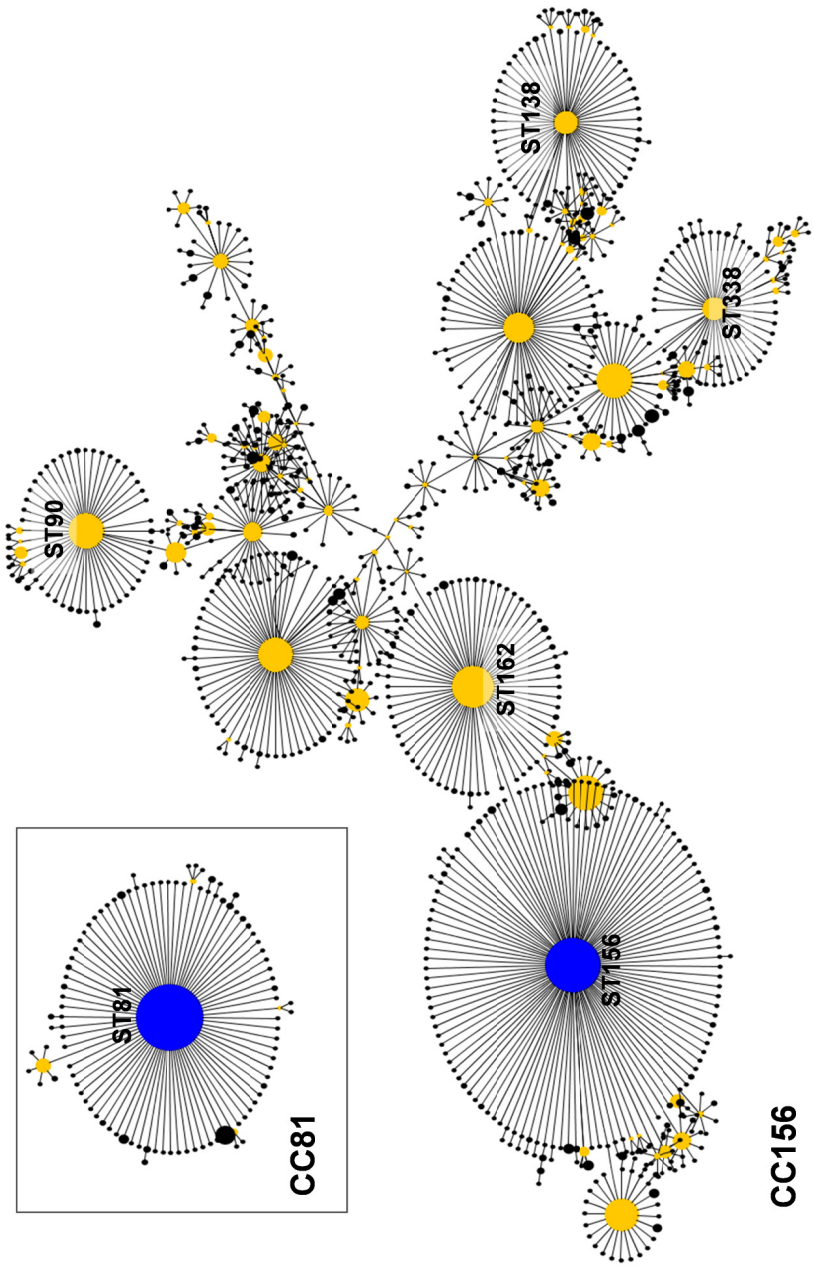

Figure 4. The genetic lineages CC81 (upper left) and CC156 as defined by the eBURST algorithm show the penicillin-resistant Pneumococcal Molecular Epidemiology Network (PMEN) clones PMEN1 Spain^{23F}ST81 and PMEN3 Spain^{9V}ST156. CC156 has expanded to include the PMEN2 Spain^{6B}ST90 clone. The predicted primary founders of the clonal complexes (CCs) are displayed in blue, subgroup founders in yellow, and each node in the diagram represents a single locus variant of any genotype to which it is connected. The size of a node is relative to the number of isolates of the genotype in the database [75, 227].

2.7 Studying the pneumococcal population by serotyping

Before the era of antimicrobial resistance and serotype-specific vaccines, serotyping was not considered to be of high importance, as all serotypes were susceptible to the available antimicrobial agents [188]. As antimicrobial resistance developed and displayed serotype dependent associations, there was an increasing interest in characterising pneumococci further. All current vaccines offer protection against disease caused by a selection of serotypes, making serotype-based surveillance essential today. Serotyping also provides information about strain relatedness in outbreak situations and the serotype can be viewed as a clonal marker [124, 136]. Serotyping traditionally refer to grouping pneumococci by the immunological properties of their capsule, but lately, serotype deduction based on the genotype of the isolate has become widely used.

2.7.1 Phenotypic serotyping methods

Quellung, or the capsular reaction test, is the classic reference test for serotyping pneumococci. It is still performed largely in the same way as it was first described in 1902, by mixing together broth culture of the strain to be studied and rabbit antisera on a microscope slide. The resulting reaction is observed in a phase contrast microscope. A positive reaction is marked by swelling of the capsule and frequently also agglutination of the bacterial cells (Figure 1) [12, 131]. Quellung is still much in use, but it requires a large panel of antisera, plenty of experience, and the individual handling of isolates.

Other phenotypic serotyping techniques include latex or co-agglutination [224], counterimmunoelectrophoresis (CIEP) [133], dot blot [102], and Fourier-transform infrared spectroscopy with chemometrics [319]. Highly multiplexed flow cytometry-based techniques using monoclonal and polyclonal antibodies have also been developed [182, 341, 342]. While the newer phenotypic methods all have their distinct benefits and often surpass Quellung in terms of rapidity and cost, some of the methods require sophisticated and expensive instruments. On the other side of the spectrum, the agglutination methods often rely on an array of self-prepared reagents [224], while in CIEP, the neutral serogroups 7, 14, 33, and 37 fail to produce precipitation lines [133].

2.7.2 Deducing the serotype by detecting the *cps*

In recent years, several DNA-based techniques have been developed to facilitate serotype deduction from the genetic properties of the isolate. The PCR-based techniques are multiplexed amplification schemes targeting the genes that encode the enzymes required for the production of each serotype. The amplification and identification of the products is performed by multiplex PCR (mPCR) and gel

electrophoresis [240], by real-time mPCR [257], by reverse line-blot hybridization [167], or by mPCR with labelled oligonucleotide primers through fluorescent fragment size analysis [280] and capillary electrophoresis [177]. Because of sequence similarity between serotypes, certain serotypes or serogroups are co-detected in PCR. In these cases, the results should be verified and subtyped by Quellung or other phenotypic serotyping methods.

Serotyping based on sequencing, sometimes called molecular capsular typing, sequetyping, or capsular sequence typing, rely on partially sequencing the integral membrane regulatory protein gene *wzg* and the capsule biosynthesis protein-tyrosine phosphatase gene *wzh*, or partially or completely sequencing only the latter. In most serotypes, these two genes were previously denominated *cpsA* and *cpsB*, respectively. The obtained sequence is used to interrogate either a public genetic sequence database, such as GenBank [302], or a database set up for the purpose of serotyping pneumococci. An identical sequence in the database or the closest match gives the serotype of the isolate [87, 169, 180]. Phenotypic methods should be employed for the verification of all novel DNA sequences that do not yield an exact match.

All nucleotide sequence-based serotyping schemes are by definition indirect, as they detect genes that encode for the proteins that synthesise the capsule, rather than the capsule itself. Researchers that discovered serotypeable isolates among strains that did not carry the expected *cps* genes caution against deducing the serotype of isolates without screening all *cps* genes [275]. An advantage of *cps*-based serotype deduction is that it enables typing of clinical samples rather than only cultured stains [124]. As nucleotide based methods have been increasingly employed, novel subtypes and features of the *cps* locus have been described and the diversity of the locus has been revealed. Upon closer study, it appears that the reference strain for serotype 22F published in 2006 contains the glycosyl- and acetyl-transferase genes *wcwA* and *wcwC* that are not present in all serological 22F strains [275]. Similarly, serogroups 6, 19, and 20 show sequence diversity. In the case of serogroup 20, this has led to the characterisation of a novel subtype, while serogroup 6 now comprises seven different proposed serotypes [42, 85, 164, 237]. Variations in the *cps* locus also include the proposed serotype 33E and genes present in a variety of non-typeable presumably non-encapsulated isolates [275]. By verifying the species prior to serotyping or *cps*-sequencing, closely related species are excluded. In rare cases, other alpha-haemolytic streptococci may carry *cps*-like genes [45].

2.8 Studying the pneumococcal population by genotyping

The three first complete pneumococcal genome sequences were published in 2001 and were obtained from virulent serotype 4 and 19F isolates and the commonly used avirulent non-encapsulated laboratory strain R6 [81, 142, 301]. Since then, several hundred pneumococcal whole-genome sequences have been completed or pursued to the draft level. These cover further non-encapsulated isolates and at least 30 different serotypes including clinically important serotypes 3, 5, 6B, 9V, 14, 19A, 19F, and 23F [65, 80, 139, 302].

The single pneumococcal genome consists of a circular chromosome, which is 2.0 to 2.3 Mb in size (Figure 5). It has an average of just over 2,200 open reading frames and a GC content of 39.7%. On average, 74% of the pneumococcal genome is shared by all strains [80]. A core genome, consisting of just under 1,200 genes that are present in a single copy in all studied pneumococcal genomes, has been identified [65]. According to the so-called distributed-genome hypothesis, bacteria possess a supragenome, or pan-genome, which exceeds the genome size of any individual bacterium, but allow the species access to dispensable genes outside the core genome. These genes are utilised by the pneumococcus through genetic recombination as a means of diversity [140]. The pneumococcus is able to incorporate genes from other species as well, and *Streptococcus mitis* appears to be the main reservoir for pneumococcal genetic diversity [80].

While studies have shown that the capsule and serotype are the most important factors in pneumococcal virulence, the issue is far from simple. In some capsular switching experiments, the disease outcome has not been associated with the serotype, instead possibly indicating the genetic background of the stain as an important factor [205, 213, 285, 328, 329]. As with pneumococcal serotypes, certain genotypes are also more associated with invasive disease and others with carriage [71, 121]. To further complicate matters, there is also intraclonal variation between different strains of the same genotype [36]. In some studies, there is a strong association between serotype and genotype [39], but others found that for most serotypes, frequent recombination events disrupt the associations between genetic lineages and serotypes [80].

Complete genome sequencing is the most thorough method of genetic analysis of a bacterial strain. However, several less costly and less labour-intensive standardised genotyping methods are generally used to provide a picture of the genetic background of the strain and its relationship to other known strains. These may be used for outbreak investigations, surveillance purposes, as well as for the determination of possible capsular switching events. Pulsed-field gel electrophoresis (PFGE) is a method in which the bacterial genome is digested with a rarely cutting

restriction enzyme and the resulting large DNA fragments are separated in an agarose gel with a pulsing field rather than a continuous field. The changing direction of the field facilitates separation of large DNA fragments and the resulting band profile is analysed with the help of computer software and compared to previously typed isolates [304]. While PFGE is a type of reproducible whole genome analysis, there is little consensus when it comes to profile naming and it is unclear whether the same sized bands in two strains actually represent the same piece of the genome [124].

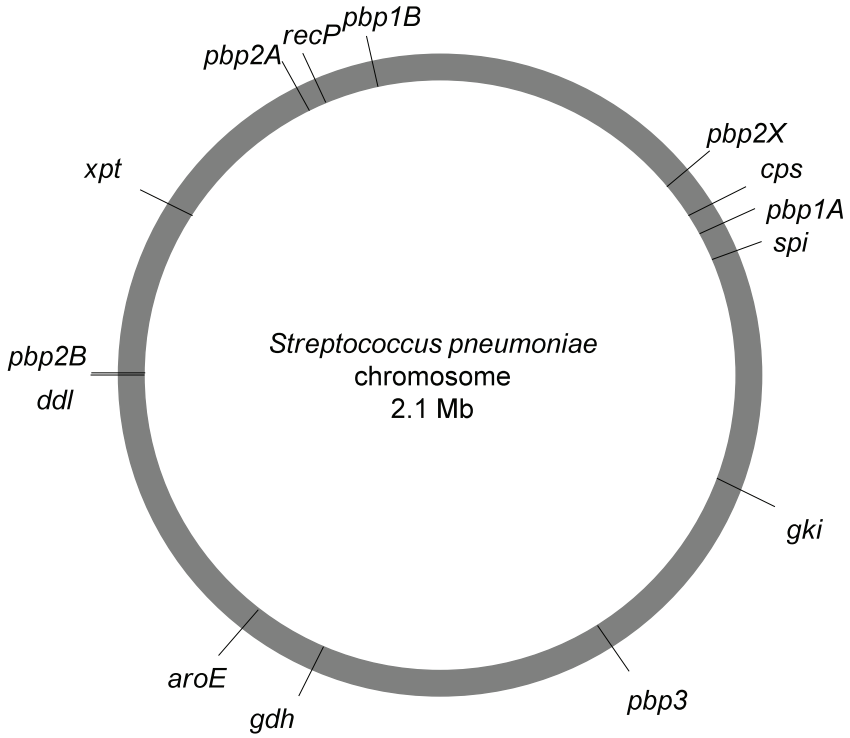

Figure 5. A schematic drawing of the pneumococcal chromosome. The positions of the capsule polysaccharide synthesis locus (*cps*), multi locus sequence typing genes (*aroE, gdh, gki, recP, spi, xpt, ddl*), and penicillin-binding protein genes (*pbp*) are displayed. Adapted from Brueggemann *et al,* 2007 [37].

Multi locus variable number tandem repeat analysis (MLVA) makes use of repeat elements such as the so-called BOX elements, which are highly conserved repeat elements on the pneumococcal chromosome. Depending on the scheme, eight or sixteen repeat elements are amplified through simplex or multiplex PCR and sized to determine the number of repeats [86, 166].

2.8.1 Multi locus sequence typing

Multi locus sequence typing (MLST) relies on the amplification and sequencing of fragments of housekeeping genes located in the bacterial genome. The genes are chosen to represent variation that arises slowly over time and is selectively neutral, as the genes are vital for the survival of the bacteria. For the pneumococcal MLST scheme, internal fragments of about 450 bp each of seven genes were chosen [90]. The genes encode shikimate dehydrogenase (*aroE*), glucose-6-phosphate dehydrogenase (*gdh*), glucose kinase (*gki*), transketolase (*recP*), signal peptidase I (*spi*), xanthine phosphoribosyl transferase (*xpt*), and D-alanine-D-alanine ligase (*ddl*) (Figure 5). The obtained sequences of these loci are compared to the material deposited in the pneumococcal MLST database, which is located at Imperial College London and can be accessed over the internet [227]. Database interrogation yields a numeric allelic profile, e.g. 7-11-10-1-6-8-1, in which each number represents an allele of the seven housekeeping genes. This string is collectively called the sequence type (ST) of the strain and is identified by a unique number, e.g. ST156. The STs can further be assembled into genetic lineages or clonal complexes (CCs) through the based upon related sequences (eBURST) algorithm [75]. Each CC includes STs that are descended from a predicted primary founder ST, after which the CC is named, e.g. CC156. Submission of information about any genotyped isolate to the database is encouraged, and all novel sequences are submitted to the database curator for verification and inclusion in the sequence collection. In September 2013, the pneumococcal MLST database contained 20,867 isolates and 9,061 unique STs [227].

MLST is currently the most widely used method for genotyping pneumococci. Its benefits include uniform and unambiguous nomenclature and interpretation, as well as the ease of comparing sequences and results between laboratories. The MLST database is accessible to anyone with access to the internet. The database is curated, which ensures the high quality of the deposited information [227]. Interpretation of MLST does not vary depending on the situation or source material, while different rules for PFGE interpretation may need to be implemented depending on the setting [304]. In an outbreak situation, PFGE may offer some advantages, as is a more discriminating method than MLST, which in turn, is either more or less discriminating than MLVA, depending on the scheme used [86, 311]. Genotyping by MLST corresponds well with whole-genome studies by microarrays and offers a good view of the genetic content of the strain, something that serotyping does relatively poorly [71]. MLST is also compatible with whole-genome sequencing, as the complete chromosomal sequence includes the housekeeping gene sequences used for MLST [124, 272]. To allow further discrimination between strains and bridge the gap between full genome sequencing and MLST, an expanded MLST

scheme has recently been developed. This is based on sequencing 96 variable loci flanked by conserved regions identified in the pneumococcal core genome [62].

2.9 Natural genetic transformation

The ability to take up free DNA from the environment and through homologous recombination incorporating it into its own genome through transformation, i.e. natural competence, is an important characteristic of the pneumococcus [80]. In addition to the original observation of transformation of capsular synthesis genes, transformation of genes involved in other biochemical pathways, such as resistance to antimicrobial agents has been documented [65, 66, 337]. Transformation has led to marked diversity in pneumococcal populations and the abundance of mosaic genes in the pneumococcal genome [154]. The evolution of the pneumococcus is mainly brought about by the horizontal genetic exchange between other pneumococci and other species sharing the same ecological niche [80]. It has been estimated that a pneumococcal nucleotide site is approximately fifty times more likely to change through recombination than through mutation, and pneumococcal alleles are changed ten times more frequently by recombination than by mutation [100]. In a collection of PMEN1 Spain^{23F}ST81 isolates, nearly three quarters of the genome has been found to be affected by recombination events [66]. Further, this capacity for natural transformation has made the pneumococcus one of the most important model organisms for studying horizontal gene transfer through natural transformation in bacteria [129].

While natural transformation is a normal part of the physiology of the pneumococcal species, the bacteria are not constantly in a state of competence [129]. In laboratory cultures, competence is spontaneously achieved during the early logarithmic growth phase and lasts for up to an hour [24]. In pneumococcal biofilm communities, which are a form of sessile organisation found in nasopharyngeal carriage, the pneumococci show efficient transformation capacity compared to free planktonic growth. This may have important implications for the spread of resistance factors and capsular genes in the pneumococcal population, especially as co-colonisation with two different strains in the nasopharynx appears to increase the fitness and colonising capacity of the pneumococci [195, 196]. Attached pneumococci are also more resistant to antibiotic treatment than planktonic cells [195].

The genes required for natural transformation are dispersed throughout the genome [129] and are co-regulated with lytic enzymes. The co-regulation has been explained by the fact that pneumococci engage in so-called fratricide, or sibling killing, in order to make naked DNA available. In fratricide, bacteria in the competent state produce and emit fratricins, muralytic enzymes that lyse bacterial cells. The predator

bacteria protect themselves against the bacteriolytic fratricins, while directing these means against non-competent target pneumococci [24]. Upon the death of the target bacteria, their DNA is released into the environment making it accessible to the predator bacteria [24, 54]. In addition to facilitation and acquisition of DNA uptake, the fratricins seem to provide a way of waging chemical warfare to eradicate competing bacteria and using the free DNA from lysed bacteria to promote genetic variation. The DNA also provides structural support in biofilm formation, which in turn helps protect the pneumococci from phagocytosis and complement mediated clearance [24, 79, 326].

Capsular switching is one of the consequences of pneumococcal natural competence [14, 113]. Capsular switching refers to a pneumococcus of one genetic background taking the capsular type of another strain, by the transfer of *cps* genes and has been described in several genotypes and serotypes [154]. The other properties of the strain such as the antibiotic resistance profile and the genotype remain unaffected, unless the genes involved are included in the transferred DNA sequence [232]. Capsular switching may result in more effective evasion of the immune system and in increased virulence [66, 232]. When isolates of different serotypes co-exist for example in the nasopharynx, capsular switching and other forms of recombination may occur [196]. Capsular switching has been occurring regularly over the past seven decades, predating strong selection pressure asserted by the use of either antimicrobial agents or vaccines [336]. Capsular switching events may include recombination of *cps* flanking regions, in which case the PBP encoding genes *pbp1A* and *pbp2X* can be altered; however, the mean length of the recombination sequence is 2.3 kb [66, 336]. Antimicrobial induced pressure also appears to encourage pneumococcal transformation. The genes required for natural competence are activated by the presence of fluoroquinolones and aminoglycosidases and this may result in a resistant genotype [263].

Several of the PMEN clones have serotype variants, where the genetic profile and ST is the same as the original strain, but the serotype is different, indicating that the clone has gone through capsular switching [67, 154, 227]. Within the PMEN3 Spain^{9V}ST156 clone, switching from serotype 9V to serotype 14 has occurred at least twice and has been demonstrated through thorough experiments [57]. In the long term, the *cps* locus has undergone horizontal transformation between clones, but in the shorter term clonal expansion is likely to be more common than capsular switching, if the frequency of a serotype increases. This is illustrated by the association of the serotype and the genotype [38, 39, 90]. Reversely, in the MLST database, identical housekeeping gene alleles can be identified in isolates from differing CCs [75, 227], suggesting that the alleles may have relocated from one strain to another during natural transformation events. The *ddl* locus is usually

omitted from phylogenetic analysis because of its propensity to be involved in horizontal gene transfer events [89, 124, 227].

2.10 From whole-cell vaccines to polysaccharide and conjugate vaccines

In the early 1900s, crude whole killed pneumococcal cells were used experimentally for immunisation and some efficacy against pneumonia could be seen [49]. When the capsular polysaccharide could be extracted in 1926, the ease of inoculation improved and led to successful vaccination studies in the 1930s and 1940s [193, 325]. A hexavalent vaccine was licensed for human use following World War II, but in the advent of antimicrobial drugs the vaccines were neglected and eventually withdrawn [325].

The idea of vaccines as a means of preventing pneumococcal disease came back into favour in the 1960s after the appearance of antimicrobial resistance and the research into vaccine research was revived [193, 325]. In 1983, a 23-valent polysaccharide vaccine containing capsule polysaccharides of the most prevalent serotypes was introduced and it is still available today and useful for preventing disease among the elderly and in defined clinical risk groups [193, 215, 299].

Already in the late 1920s, researchers discovered that linking the pneumococcal capsular polysaccharides to foreign carrier proteins increases the obtained antibody response. Later on, this technology was used successfully to create a vaccine against another capsulated bacterial pathogen, *H. influenzae* type b, and in the past decades for the development of pneumococcal conjugate vaccines (PCVs) [193].The conjugate vaccines elicit a high immune response also in young children, the elderly, and the immunocompromised, unlike the polysaccharide vaccines [332]. The carrier protein in the PCV enables the activation of T-cells in eliciting an immune response and induces an immunological memory, something the polysaccharide vaccine fails to do [260].

The first pneumococcal conjugate vaccine was launched in the year 2000 in the United States and in 2001 in Europe. This heptavalent conjugate vaccine (PCV7) contains polysaccharides of serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F [327]. Since then, two other PCVs have been developed. In 2009, the 10-valent conjugate vaccine (PCV10), covering serotypes 1, 5, and 7F in addition to the PCV7 serotypes, was launched [241, 291]. A year later in 2010, an expanded version of PCV7 was introduced. This 13-valent conjugate vaccine (PCV13) contains antigens of serotypes 3, 6A, and 19A, in addition to the PCV10 serotypes [327]. The use of PCVs has successfully decreased the invasive disease incidence in areas with high

vaccine coverage. Among young children, the decrease has often been rapid and dramatic [9, 74, 82, 145, 150, 152, 212, 255, 271, 283, 331]. As of December 2012, 86 (44%) of the WHO member states have introduced a PCV into their national vaccination programmes [47]. Low- and middle-income countries have not introduced PCVs in the same degree as high-income countries, although this is where most of the deaths from pneumococcal disease take place [158]. As a result, 69% of the children born in the WHO member states are still ineligible for vaccination [47]. In Finland, the 10-valent conjugate vaccine has been part of the national vaccination programme since 1st September, 2010. It has since been offered free of charge to all infants born as of 1st June, 2010, at a 2+1 schedule, with immunisation at 3, 5, and 12 months of age [299]. Prior to universal childhood vaccination, a cluster-randomised PCV10 trial was conducted in Finland in the years 2009 to 2010 [241]. Already in the first few years following implementation of PCV10 vaccination in the national vaccination programme, the number of invasive pneumococcal disease cases among young children has decreased [269].

2.11 Intervention and the pneumococcal population

Both antimicrobial treatment and large-scale vaccination campaigns are interventions that affect the pneumococcal population as a whole. As resistance to commonly used antimicrobials such as penicillin and erythromycin is clonal, the consumption of antimicrobials favour the resistant clones, which may be allowed to acquire even further resistance determinants [60, 291, 312] Some studies have indicated that a decrease in the consumption of antimicrobials leads to a decline in resistance, but this is not always the case [6, 7]. Clonal spread of resistant lineages may reduce the impact on resistance levels of decreased consumption of antibiotics [7, 291]. Furthermore, large-scale vaccination and changes in the overall consumption of antimicrobials may have synergistic or conflicting effects on the resistance rates in a society [291].

The success of the PCVs, at least in the USA, is a combination of herd immunity and individual protection conferred by the vaccine and both carriage and invasive disease are affected by the vaccine [153, 327]. The use of a serotype-dependent vaccine will inevitably assert selection pressure on the pneumococcal population and it will lead to a decrease in vaccine serotype coverage, as the serotypes covered by the vaccine will be eliminated or decrease in the bacterial population. Another effect of the vaccines includes the so-called serotype replacement, in which non-vaccine serotypes gain from the decrease of vaccine serotypes by increasing in prevalence [212, 294, 327]. In the USA, the reduction in invasive disease was sustained in the seven years following PCV7 introduction [255], however, the positive effect of the vaccine may in time be partially eroded because of serotype replacement. As with

pneumococcal pathogenesis, serotype replacement in disease begins with colonisation. The incidence of non-vaccine serotypes have increased in carriage following vaccination [151, 327]. It has been demonstrated that PCV7 reduces the rates of colonisation with vaccines serotypes, but simultaneously increases the risk of non-vaccine serotype isolate acquisition [235] and the colonisation prevalence as a whole may not decrease following vaccination [148, 149]. There is indication of serotype replacement also in invasive disease [138, 146, 212, 228, 295]. For example, in France, the overall pneumococcal meningitis incidence in children returned to pre-vaccination levels after five years of PCV7 use, because of replacement primarily by serotypes 19A and 7F [181]. Capsular switching events allow the established clones circulating in the population to adapt to the post-vaccine environment by changing their serotype to a non-vaccine type, such as a switch from serotype 4 to serotype 19A in the PCV7 era [4, 37].

Initially, antimicrobial resistance rates were expected to decrease following PCV use. Some studies report significant reductions in resistance, however, the results from different studies are contradictory and the long-term impact is still not clear [70, 206, 228, 282]. Among carriage isolates, penicillin-resistance increased in one study due to non-PCV7 serotypes [120] and according to another study, invasive pneumococci are more likely to be non-susceptible to penicillin in the PCV7 era compared to the pre-PCV7 era [162]. The potential decrease in antimicrobial resistance has been further complicated by the emergence of multidrug-resistance as a feature of serotype replacement, mainly through the expansion of established multidrug-resistant clones [21]. Serotype 19A is a frequently observed replacement serotype [212], and is often resistant to several antimicrobial agents. Among the PCVs, serotype 19A is covered only by PCV13 [70]. In the USA, the incidence of invasive 19A disease increased following vaccination, while the resistance within this serotype increased markedly due to expansion of multidrug-resistant clones [21]. In Norway, where the antimicrobial selection pressure is low, the increase in invasive serotype 19A disease is caused mainly by penicillin-susceptible isolates [320].

In addition to changes in the serotype distribution, the vaccine may also indirectly affect the epidemiology, because the serotypes vary in invasive potential and the diseases caused. While the incidence of parapneumonic empyema has been increasing worldwide even before PCV use, the emergence of non-vaccine serotypes has also had an impact on this complication [40, 174]. Following PVC-7 vaccination, the incidence of paediatric pneumococcal parapneumonic empyema increased in Utah, USA, probably due to replacement by non-vaccine serotypes such as serotype 1 [41]. A similar situation among adults was described in Spain, where a virulent serotype 1 strain caused pneumonia with empyema after PCV7 implementation [110]. Non-PCV7 serotypes 1, 3, and, 19A are especially prone to causing empyema

[40]. Other non-vaccine serotypes, such as 22F and 33F, have been found to have a particular propensity to cause meningitis [315].

It has been suggested that the pneumococcal vaccine strategy is at risk of Red Queen dynamics [153], a situation named after the character in *Through the Looking-Glass* by Lewis Carroll, who said "It takes all the running you can do, to keep in the same place" [44]. In evolutionary biology, Red Queen dynamics illustrate an arms race, in which a pathogen and its host organism co-evolve, leading to a situation in which their relative position to one another remains unchanged [191]. In the case of the pneumococcus and its human host, the vaccine development and improvement represents the evolution of the host, while serotype switching and serotype replacement represents the pneumococcal response. Both vaccine development and changes in serotype distribution respond and co-respond to the co-evolving party [153]. There are a few vaccine development strategies for overcoming the Red Queen dynamics. Firstly, a PCV covering all known serotypes would solve the stalemate. Because of cost and technical constraints regarding the limit of carrier protein and polysaccharides in a vaccine, this approach would more likely entail a set of PCVs, each covering a set of serotypes and together cumulatively covering all known serotypes. The second approach would be the development of a pneumococcal vaccine, which would be effective against all pneumococci and independent of serotype [153, 193, 216]. Several of the protein structures protruding from the pneumococcal surface have been proposed as multivalent vaccine candidates. Among these are the pneumococcal pili encoded by PI-1 and PI-2. Immunisation with PI-1 subunits has been shown to protect mice from lethal challenge from piliated pneumococci [16, 108, 223].

3 Aims

The general aim of this study was to examine the clonality of the invasive pneumococcal population both on the serotype and the genotype level, focusing especially on pneumococcal clonality in relation to antimicrobial susceptibility. For a more comprehensive view of drug-resistant pneumococcal population, the clonality of a subset of multidrug-resistant non-invasive isolates was also examined. By combining clonal and virulence factor analysis, this study may be useful when future prevention strategies are considered and developed.

The specific aims of this study were:

- to study the clonality by serotyping and genotyping, as well as the antimicrobial susceptibility trends among the invasive pneumococcal population in Finland in the years 2002 to 2011 (Publications I and IV)

- to study the clonality of a subset of multidrug-resistant non-invasive isolates (Publication II)

- to set up and validate a serotyping scheme suitable for serotyping the invasive pneumococci in Finland (Publication III)

4 Materials and methods

4.1 Bacterial isolates

Table 2 presents the number of isolates included in the individual publications, the years of isolation, type of specimen and the collection or source of the isolates, as well as the number of isolates studied by the main methods. All isolates were stored in skim milk vials at below -65°C until needed and cultivated on 5% sheep blood agar at +37°C in an atmosphere enriched with 5% CO_2.

The publications I and IV include the pneumococcal isolates recovered from blood or CSF in Finland in 2002 to 2011 and collected in the Culture Collection of the NIDR housed at THL were studied. The isolates for which unpublished genotyping results are shown were intermediately penicillin-resistant pneumococci isolated in the year 2005.

Based on previous results, positive control strains for species verification, the serotyping methods, and the PCR methods detecting macrolide resistance determinants and pilus-encoding genes were selected from the culture collections of THL, unless otherwise mentioned. For antimicrobial susceptibility testing, *Streptococcus pneumoniae* (ATCC® 49619™) was used for quality control.

4.2 Species identification

Verification of the bacterial species was performed primarily by colony morphology and by optochin sensitivity testing using optochin disks (Rosco Diagnostica, Taastrup, Denmark) [33]. When needed, the bile solubility test was performed by mixing bacterial culture in 10% sodium deoxycholate and noting lysis of the cells. If needed, the *lytA* gene was detected by PCR to confirm the species using a *Taq* polymerase (Ampli*Taq* Gold, Applied Biosystems, Foster City, CA, USA, or Maxima Hot Start, ThermoScientific, Waltham, MA, USA) and an annealing temperature of 60°C (Table 3). The Primer Basic Local Alignment Search Tool (Primer-BLAST) was used for primer design [303].

4.3 Isolation of DNA

The genomic DNA of the bacterial strains was isolated either with the help of a column kit DNeasy Tissue kit (QIAGEN GmbH, Hilden, Germany) (Publication I) or by a heat lysis method in TE buffer containing Triton X-100, 1 % and Tween 20, 0,5% (Publications I-IV) [214].

Table 2. The number of isolates included in the individual publications, the years of isolation, specimen type, the collection or source of the isolates, and the number of isolates studied by each of the main methods.

Publication		Isolates (*n*)	Year(s) of isolation	Specimen type	Collection and further information	Serotyping (*n*)	Antimicrobial susceptibility testing (*n*)	MLST (*n*)	Rationale for MLST subset
I		3,571	2002-2006	invasive	NIDR Culture Collection	3,571	3,571	88	PEN MIC≥2 mg/L
II		12	2008	non-invasive	HUS	12	12	12	multidrug-resistance
III									
Validation set	**1**	70	2009	invasive	consecutive NIDR Culture Collection isolates	70	-	-	-
	2	70	unknown	unknown	EQA isolates from SSI [170]	70	-	-	-
	3	30	2009	invasive	serogroup 6 NIDR Culture Collection isolates	30	-	-	-
Routine use		1,304	2010-2011	invasive	NIDR Culture Collection	1,304[a]	-	2	discrepant serotyping results
IV		4,194	2007-2011	invasive	NIDR Culture Collection	4,194	4,194	12	PEN MIC ≥4 mg/L
Unpublished, Siira *et al.*		50	2005	invasive	NIDR Culture Collection	50[b]	50[b]	50	PEN MIC 0.12-1 mg/L

MLST, multi locus sequence typing; NIDR, National Infectious Disease Register; HUS, Hospital District of Helsinki and Uusimaa; SSI, Statens Serum Institute; EQA, external quality assurance; PEN, penicillin; MIC, minimum inhibitory concentration; [a] also included in Publication IV; [b] also included in Publication I.

Table 3. Oligonucleotide primers used in the study.

Method	Target gene(s)	Primer sequence (5'-3')	Publication	Reference
Species verification	*lytA*	TGGGGGCGGTTGGAATGCTG	III	This study
	lytA	CCAGCCTGTTCCGTCCGCTG	III	This study
MLST	*aroE*	CGTTTAGCTGCAGTTGTT GC	I-IV	[88]
	aroE	CCCACACTGGTGGCATTAAC	I-IV	[88]
	gdh	ATGGACAAACCAGCNAGYTT	I-IV	[90]
	gdh	GCTTGAGGTCCCATRCTNCC	I-IV	[90]
	gdh	CTACAACTTCTTTCGCTCCT	IV	[27]
	gdh	TGCCAAGTCCATTTGGCACC	IV	[27]
	gki	GGCATTGGAATGGGATCACC	I-IV	[90]
	gki	TCTCCCGCAGCTGACAC	I-IV	[90]
	recP	GAATGTGTGATTCAATAATCACC	I-IV	[88]
	recP	TTCGATAGCAGCATGGATGG	I-IV	[88]
	spi	CGCTTAGAAAGGTAAGTTATG	I-IV	[259]
	spi	AGGCTGAGATTGGTGATTCTC	I-IV	[259]
	xpt	CCACTACAACGGGAAATATTTGA	I-IV	[88]
	xpt	AGGATAGATCCTGAGTACATG	I-IV	[88]
	ddl	TTGCCATGGATAAAATCACGAC	I-IV	[250]
	ddl	CGCGCTTGTCAAAACTTTCC	I-IV	[27]
	ddl	AGCGTGTTCTGGAATCTGC	IV	[28]
	ddl	AGGTCAACCAAACGCTCG	IV	[28]
macrolide resistance determinant detection	*erm*(B)	GAAAARGTACTCAACCAAATA	I, II	[103]
	erm(B)	AGTAAYGGTACTTAAATTGTTTAC	I, II	[103]
	erm(A)	CTTGTGGAAATGAGTCAACGG	I, II	[253]
	erm(A)	TTGTTCATTGGATAATTTATC	I, II	[253]
	mef(A/E)	GGCAAGCAGTATCATTAATCAC	I, II	[103]
	mef(A/E)	GACTGCAAAGACTGACTATAG	I, II	[103]
	mef(A)	TGGTTCGGTGCTTACTATTGT	I	[72]
	mef(A)	CCCCTATCAACATTCCAGA	I	[72]
	mef(E)	GGGAGATGAAAAGAAGGAGT	I	[72]
	mef(E)	GCTATAAAATGGCACCGAAAG	I	[265]
PI-1 detection	*rlrA*	TCTGATAGATGAGACGCTGTTG	I, II, IV	[2]
	rlrA	CTCCGCTTCTTTCTACTACAA	I, II, IV	[2]
	rrgC	GACTTCGTTTGAGATGACCCTTC	I, II, IV	[284]
	rrgC	GTCGTAACAGCATAGCCTGC	I, II, IV	[284]
PI-2 detection	*pitA-sipA*	CGTGGGTATCAGGTGTCCTATGATAA	II, IV	[16]
	pitA-sipA	GCCTCGTCTTCTAATGACTGTTAC	II, IV	[16]

(Table 3. continues)

Method	Target gene(s)	Primer sequence (5'-3')	Publication	Reference
19F-like	*wzy*	CTTGTTTTCAAAYAAATGCAACTTT	III	This study
19A *wzy*	*wzy*	AGATTTGAAGAAATRACATAAGARAAA	III	This study
sequencing	*wzy*	GACGTATCTGCTTATATTTTTGGATATA	III	This study
	wzy	CCTCTATCCGTGTTCTTATCCGTGT	III	This study
	wzy	AAGAGTAGTAAAACGCTGGAATACCA	III	This study

MLST, multi locus sequence typing; PI-1, pilus islet 1; PI-2, pilus islet 2

4.4 Serotyping

The serotyping of all studied pneumococci isolated before 2010 was performed using phenotypic antisera-based methods, i.e. latex agglutination, CIEP, the Quellung reaction test at a THL laboratory in Oulu (Publications I-IV). The serotyping of pneumococci isolated since 2010 was performed using an mPCR-based scheme supplemented when needed by the Quellung reaction at a THL laboratory in Helsinki (Publication III-IV).

The serotyping for the verification of the validation results (Publication III) was performed by Quellung at Statens Serum Institute (SSI), Denmark, according to instructions from the antisera manufacturer SSI Diagnostica, and by a monoclonal antibody based assay at the University of Alabama (UAB), Birmingham, USA [341].

The distinction of serotype 6C from serotype 6A has been possible since late 2009, but was performed retrospectively for the years 2007-2009; however, for the ten year study period these two serotypes were pooled together for analysis. The serotypes 10A and 10F/C were distinguished as of the year 2010, these serotypes were analysed at the serogroup level.

4.4.1 Latex agglutination

The neutral serogroups were identified by latex agglutination using antisera (SSI Diagnostica, Copenhagen, Denmark) and latex beads (Difco Laboratories Inc, Detroit, MI, USA). The latex antisera reagent was mixed together with the pneumococcus to be studied and observed for an agglutination reaction [179].

4.4.2 Counterimmunoelectrophoresis

Apart from the neutral serogroups, serotypes isolated prior to 2010 were identified primarily by CIEP using omni, pool, group or type, and factor sera (SSI Diagnostica). The pneumococci to be studied were harvested from overnight plate cultures, suspended in physiological NaCl solution, and heated in a water bath. After

cooling, droplets of suspension and antisera were placed in separate wells on a counterimmunoelectrophoresis gel and run in a counterimmunoelectrophoresis chamber [179]. Each gel was examined twice for precipitation lines between antisera and pneumococcal suspension. The 23-valent Pneumovax vaccine (Merck, Haarlem, Netherlands) was used as a positive control.

4.4.3 The Quellung reaction

The Quellung reaction was applied for serotyping, subtyping and confirmation, when needed. Fixed broth culture was with antisera (SSI Diagnostica) mixed on a microscope slide and the reaction observed in a phase contrast microscope. Physiological NaCl solution instead of antisera was used as a negative control. This method was used at the THL laboratories in Oulu (Publications I-IV) and in Helsinki (Publications III-IV), as well as at SSI for the verification of discrepant serotyping in the validation of mPCR-based serotyping (Publication III).

4.4.4 Immunologic monoclonal antibody based serotyping assay

Pneumococcal capsular polysaccharides (American Type Culture Collection, Manassas, VA, USA) were conjugated in separate reactions to distinct Luminex MicroPlex beads (Luminex Corporation, San Antonio, TX, USA). The bead mix was incubated with a pneumococcal lysate of the strain to be serotyped and pooled monoclonal antibodies (mAbs) on a filter bottom plate. After washing the beads, bound mAbs were detected with the help of phycoerythrin conjugated goat anti-mouse immunoglobulin (BD Pharmingen, Franklin Lakes, NJ, USA) in a Bio-Plex 200 analyser (Bio-Rad, Hercules, CA, USA) that detected the fluorescence signals. The obtained data was normalised to remove noise and determine the serotype of the isolate [341].

4.4.5 Serotype deduction by multiplex PCR

Setting up the serotyping scheme

A serotyping scheme based on six consecutive mPCRs was compiled to cover 99% or the serotypes found among the Finnish invasive pneumococci by analysing the serotype distribution of 5,337 invasive isolates collected in 2002 to 2008 in Finland. Each of the six assembled sequential mPCRs detected six serotype or serogroup loci, as well as the capsular regulation gene *wzg* (*cpsA*) as an internal positive control. The most commonly occurring serotypes were included in the first two mPCRs. A separate mPCR (6C/6D mPCR) for detecting the presence of *wzg* (*cpsA*), the serogroup 6 specific *wciP*, and the 6C/6D capsule gene *wci*N_{β} was also set up according to a previously described mPCR [46]. The serotyping scheme was set up so that mPCRs would be performed until a serotype-specific amplicon was produced or if all six mPCRs had been performed without producing a serotype-specific amplicon the serotyping would be performed by Quellung, as described above.

Table 4. Oligonucleotide primers for mPCR-based serotype deduction (III, IV).

Serotype specificity	Target gene	mPCR	Reference
All*	*wzg (cps*A*)*	All	[240]
1	*wzy*	mPCR 4	[240]
2	*wzy*	mPCR 6	[68]
3	*gal*U	mPCR 2	[240]
4	*wzy*	mPCR 2	[240]
5	*wzy*	mPCR 6	[240]
6A/6B/6C/6D	*wci*P	mPCR 1, 6C/D mPCR	[240]
6C/6D	*wci*N$_{\beta}$	6C/D mPCR	[46]
7A/7F	*wzy*	mPCR 2	[68]
7B/7C/40	*wcw*L	mPCR 5	[240]
8	*wzy*	mPCR 4	[68]
9A/9V	*wzy*	mPCR 1	[68]
9L/9N	*wzx*	mPCR 1	[76]
10A	*wcr*G	mPCR 3	[240]
10C/10F/33C	*wzx*	mPCR 3	[68]
11A/11D	*wzy*	mPCR 3	[240]
12A/12B/12F/44/46	*wzx*	mPCR 3	[240]
13	*wzy*	mPCR 6	[68]
14	*wzy*	mPCR 1	[76]
15A/15F	*wzy*	mPCR 4	[240]
15B/15C	*wzy*	mPCR 2	[240]
16F	*wzy*	mPCR 4	[68]
17F	*wci*P	mPCR 6	[240]
18A/18B/18C/18F	*wzy*	mPCR 2	[240]
19A	*wzy*	mPCR 3	[258]
19F	*wzy*	mPCR 2	[240]
20	*wci*L	mPCR 5	[240]
22F/22A	*wcw*V	mPCR 1	[240]
23A	*wzy*	mPCR 3	[68]
23B	*wzx*	mPCR 6	[68]
23F	*wzy*	mPCR 1	[240]
25A/25F/38	*wzy*	mPCR 5	[240]
31	*wzy*	mPCR 5	[240]
33A/33F/37	*wzy*	mPCR 5	[240]
34	*wzy*	mPCR 5	[240]
35B	*wcr*H	mPCR 4	[240]
35F/47F	*wzy*	mPCR 4	[240]
39	*wzy*	mPCR 6	[68]

*The *cpsA* gene of some serotype 38 isolates is not amplified with these oligonucleotide primers.

Serotype deduction based on mPCR

The amplification was performed in a 25 µl reaction volume with 35 thermal cycles and an annealing temperature of 54°C using a *Taq* polymerase (Ampli*Taq* Gold, Applied Biosystems, Foster City, CA, USA; or Maxima Hot Start, ThermoScientific, Waltham, MA, USA). All oligonucleotide primer names, targets, and sources are listed in Table 4. The genomic DNA of one positive control isolate for each amplified locus in each mPCR was used for the PCR-based serotyping scheme. The PCR products were visualized by gel electrophoresis using 2% NuSieve 3:1 agarose gels (Lonza, Rockland, ME, USA) in 1×TAE buffer run for 120 to 180 minutes. As a size marker the GeneRuler 50bp Marker (ThermoScientific) was used. Because of primer cross-reactivity and *cps* sequence similarity, the Quellung reaction described above was applied to distinguish co-detected serotypes, for subtyping when needed, and for serotyping if no serotype- or serogroup-specific product was obtained in any of the sequential mPCRs. During the protocol validation process, validation set 3 was studied first by the 6C/6D mPCR, after which the Quellung reaction was applied for subtyping (Publication III).

4.5 Antimicrobial susceptibility testing

The applied breakpoints for the most important antimicrobial agents studied, the definition of multidrug-resistance, and the references for all breakpoints are listed in Table 5. When analysing the susceptibility for the whole ten year study period, the EUCAST 3.0 breakpoints were applied [93]. Antimicrobial susceptibility to penicillin, erythromycin, tetracycline, and clindamycin of all available isolates was performed by the plate dilution method at a THL laboratory in Turku (Publication I, II and IV) [252]. Susceptibility to levofloxacin was tested from August 2004 and susceptibility to ceftriaxone was tested from 2005 onwards. Additionally, the susceptibility to trimethoprim/sulfamethoxazole of the non-invasive isolates was tested (Publication II). The plates were cultivated in an atmosphere enriched with 5% CO_2.

The susceptibility to penicillin, erythromycin, clindamycin, trimethoprim/ sulfamethoxazole, and doxycycline of the non-invasive isolates was initially determined by disk diffusion test and E-test at the clinical microbiology laboratory of the Hospital District of Helsinki and Uusimaa (Publication II).

Table 5. References for the breakpoints applied in antimicrobial susceptibility testing and multidrug-resistance definitions applied in the publications.

Publication	Susceptibility breakpoints applied, minimum inhibitory concentration (MIC)		Multidrug-resistance definition applied
	Penicillin	Other antimicrobials	
I*	CLSI pre-2008 [56] - S ≤0.06 mg/L - R ≥2 mg/L	CLSI pre-2008 [56], intermediate - ERY MIC ≥0.5 mg/L - TET MIC ≥4 mg/L	Non-susceptibility to PEN, ERY, and TET
II	CLSI pre-2008 [56] - MIC ≥0.12 mg/L	CLSI pre-2008 [56], intermediate - ERY MIC ≥0.5 mg/L - TET MIC ≥4 mg/L	Non-susceptibility to PEN, ERY, TET, CLI, SXT, DOX
IV	CLSI pre-2008 [56] - S ≤0.06 mg/L - R ≥2 mg/L CLSI 2008 [55] - S ≤2 mg/L - R ≥8 mg/L EUCAST 3.0 [93] - S ≤0.06 mg/L - R ≥4 mg/L	EUCAST 3.0 [93], intermediate - ERY MIC ≥0.5 mg/L - TET MIC ≥2 mg/L	Non-susceptibility to PEN (MIC≥0.12 mg/L), ERY, and TET

*Includes the isolates for which unpublished genotyping results are shown; S, susceptible; R, resistant; ERY, erythromycin; TET, tetracycline; CRO, ceftriaxone; CLI, clindamycin; SXT, trimethoprim/sulfamethoxazole; DOX, doxycycline

4.6 Detection of macrolide resistance genes

The macrolide resistance determinants of 223 randomly selected erythromycin-non-susceptible isolates (MIC ≥0.5 mg/L) (Publication I) and of 12 multidrug-resistant non-invasive isolates (Publication II) were studied at a THL laboratory in Turku. Detection of the macrolide resistance determinants *mef*, *erm*(B), and *erm*(A) was performed by mPCR (Publications I and II). Separate PCR reactions were performed to differentiate between the efflux gene subclasses *mef*(A) and *mef*(E) in the *mef* positive isolates (Publication I). The oligonucleotide primers used are presented in Table 3, and the amplification was performed using the Ampli*Taq* polymerase (Applied Biosystems).

4.7 Genotyping

Selected isolates were genotyped by MLST (Table 2) [90]. The PCR reactions were performed using a *Taq* polymerase, either Ampli*Taq* Gold (Applied Biosystems, Foster City, CA, USA) or Maxima Hot Start (ThermoScientific, Waltham, MA,

USA). The oligonucleotide primers in Table 3 were used for both PCR and sequencing reactions. PCR product clean-up was performed by QiaQuick PCR Purification kit (QIAGEN GmbH, Hilden, Germany), the GeneClean Turbo kit (Q-BioGene MB Biomedicals, OH, USA), the Edge Bio QuickStep 2 PCR Purification kit (EdgeBio Systems, Gaithersburg, MD, USA) or enzymatically using FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (ThermoScientific). Sequencing was performed using BigDye 1.3 chemistry (Applied Biosystems) either in ABI Prism Genetic Analyser (Applied Biosystems) or by full sequence service at the Institute for Molecular Medicine Finland (FIMM). The traces were analysed using either the Vector NTI Advance 10 or 11 software suite (Invitrogen Corporation, Carlsbad, CA, USA) or the Geneious Pro 5 or 6 software (Biomatters Ltd, Auckland, New Zealand) and compared with the material in the MLST database, according to which the STs were assigned [227]. Novel allele sequence traces and STs were submitted to the MLST database. Each ST was assigned to a CC by eBURST analysis using default stringent parameters [75]. The CCs were named after the predicted primary founder, i.e. the ST with the highest number of single locus variants (SLVs) at the time of writing each manuscript. For clarity and uniformity, all observed CCs were renamed after the predicted primary founder in September 2013**.**

4.8 Detection of pilus-encoding genes

The presence of pilus-1 encoding PI-1 genes (*rlrA*, *rrgC*) and pilus-2 encoding PI-2 genes (*pitA*-*sipA*) were identified by PCR in selected isolates [2, 16, 284]. The isolates were selected as follows: in Publication I, the first penicillin resistant (MIC ≥2 mg/L) isolate (n=27) of each serotype and ST as revealed by MLST, 2002-2006; in Publication II, the multidrug-resistant non-invasive pneumococci (n=12); in Publication IV, all penicillin-resistant (MIC ≥4 mg/L) isolates (n=12), 2007-2011. In Publication I, the isolates were studied only for the presence of PI-1. The annealing temperature was 59°C and the oligonucleotide primers are listed in Table 3. A *Taq* polymerase was used (Ampli*Taq* Gold, Applied Biosystems; or Maxima Hot Start, ThermoScientific).

4.9 Sequencing of serotype 19F-like serotype 19A *wzy*

The *wzy* gene of two serotype 19F-like serotype 19A isolates, which gave discrepant results by mPCR and Quellung based serotyping (Publication III), was sequenced using a primer walking strategy with the oligonucleotide primes in Table 3. The obtained sequences were edited and merged into contigs in Geneious Pro 5 software (Biomatters) and compared to the material deposited in the GenBank through the Basic Local Alignment Search Tool [303]. Finally, the sequences were submitted to GenBank.

4.10 Statistical methods

Poisson regression was used for testing the statistical significance of antimicrobial resistance trends for the years 2002 to 2006 (Publication I). For testing the statistical significance of the log-linear antimicrobial resistance and serotype distribution trends, binomial regression with log-link was used in the SPSS Statistics 21 software (IBM, Armonk, NY, USA) for the years 2007 to 2011 (Publication IV). The latter method was used for analysis of overall serotype proportion trends and non-susceptibility to erythromycin, tetracycline, and multidrug-resistance over the ten year period from 2002 to 2011. The penicillin-non-susceptibility and resistance according to EUCAST and pre-2008 CLSI breakpoints was analysed for the entire ten year period, as was non-susceptibility by serotype and age-group. The following age-groups were used: 0-2, 3-15, 16-64 and ≥65-year-olds. In the first five years of the study period, the two youngest age-groups were combined for statistical testing (Publication I). Penicillin- and erythromycin-non-susceptibility of the invasive isolates was studied by tertiary care district (Helsinki, Turku, Tampere, Kuopio, and Oulu) in five year periods (Publication I and IV). Risk ratio (RR) estimates per year and 95% confidence intervals (CI) were calculated along with *P* values.

For detecting a possible association of PI-1 or PI-2 with serotype, serogroup, or ST, statistical analysis was performed by Fisher's exact test using the Internet-based GraphPad Software (Publication II). *P* values <0.05 were considered statistically significant.

4.11 Cost analysis

Based on material usage per isolate at the time of the validation of the protocol, the cost of reagents and materials of serotyping was estimated for serotyping by Quellung and for the mPCR-based serotyping scheme including Quellung, when needed. The mean material cost of serotyping validation set 1 was considered for cost comparisons as this set best reflects the serotypes present in the Finnish invasive pneumococcal population (Publication III).

5 Results

5.1 Serotypes among invasive pneumococci, 2002-2011 (I, IV)

The proportions of the most important serotypes discovered are displayed in Figure 6A and Figure 6B. The proportion of certain serotypes increased over the ten-year period. These were serotype 14, which increased from 13.5% (82/607) in 2002 to 18.0% (143/794) in 2011 (RR, 1.03; 95%CI, 1.01-1.06; P=0.010); serotype 22F, from 3.5% to 8.8% (RR, 1.10; 95%CI 1.04-1.17; P=0.003); serotype 3 from 7.4% to 9.7% (RR, 1.04; 95%CI, 1.00-1.08; P=0.032). The proportion of serotype 4 decreased significantly from 13.6% in 2002 to 6.7% in 2011 (RR, 0.93; 95%CI, 0.90-0.96; P<0.001). The proportion of serotype 9V also decreased from 8.9% to 4.3% (RR, 0.90; 95%CI, 0.86-0.94, P<0.001).

Taken separately, the two five-year study periods showed other significant serotype changes. Between 2002 and 2006, the proportion of serotype 6B increased from 5.8% to 9.4% (RR, 1.10; 95%CI, 1.00-1.21, P=0.05), while between 2007 and 2011 it decreased from 9.2% to 5.8% (RR, 0.91; 95%CI, 0.84-0.99; P=0.036). Reversely, the proportion of serotype 22F decreased in the initial five years from 3.5% to 2.7% (RR, 0.89; 95%CI, 0.84-0.94; P<0.001), while it increased in the latter five from 4.3% to 8.8% (RR, 1.20; 95%CI, 1.06-1.37; P=0.005). The proportion of PCV10 serotypes collectively remained stable in the first five years, but decreased between 2007 and 2011 from 67.0% to 55.7% (RR, 0.97; 95%CI, 0.94-0.99; P=0.008). Serotype 6A decreased in the latter five years of the study (RR, 0.92; 95%CI, 0.86-1.00; P=0.042), while the proportion of serotype 6C did not change in a statistically significant manner.

There were significant changes in the serotype proportions in two age-groups from the year 2002 to 2011. Among the adults (16-64-year-olds), the proportion of serotype 14 increased (RR, 1.04, 95%CI, 1.01-1.07; P=0.004) reaching 20.0% in 2011; and serotype 9V decreased (RR, 0.89; 95%CI, 0.85-0.94, P<0.001) covering 5.2% of the isolates from this age-group in 2011. Among the elderly (≥65-year-olds), the proportion of serotype 22F increased (RR, 1.09, 95%CI, 1.03-1.14; P=0.002) reaching 9.4% in 2011, while serotype 9V decreased (RR, 0.91; 95%CI, 0.84-0.98, P=0.017). Among the elderly, serotype 9V covered 3.6% in the year 2011.

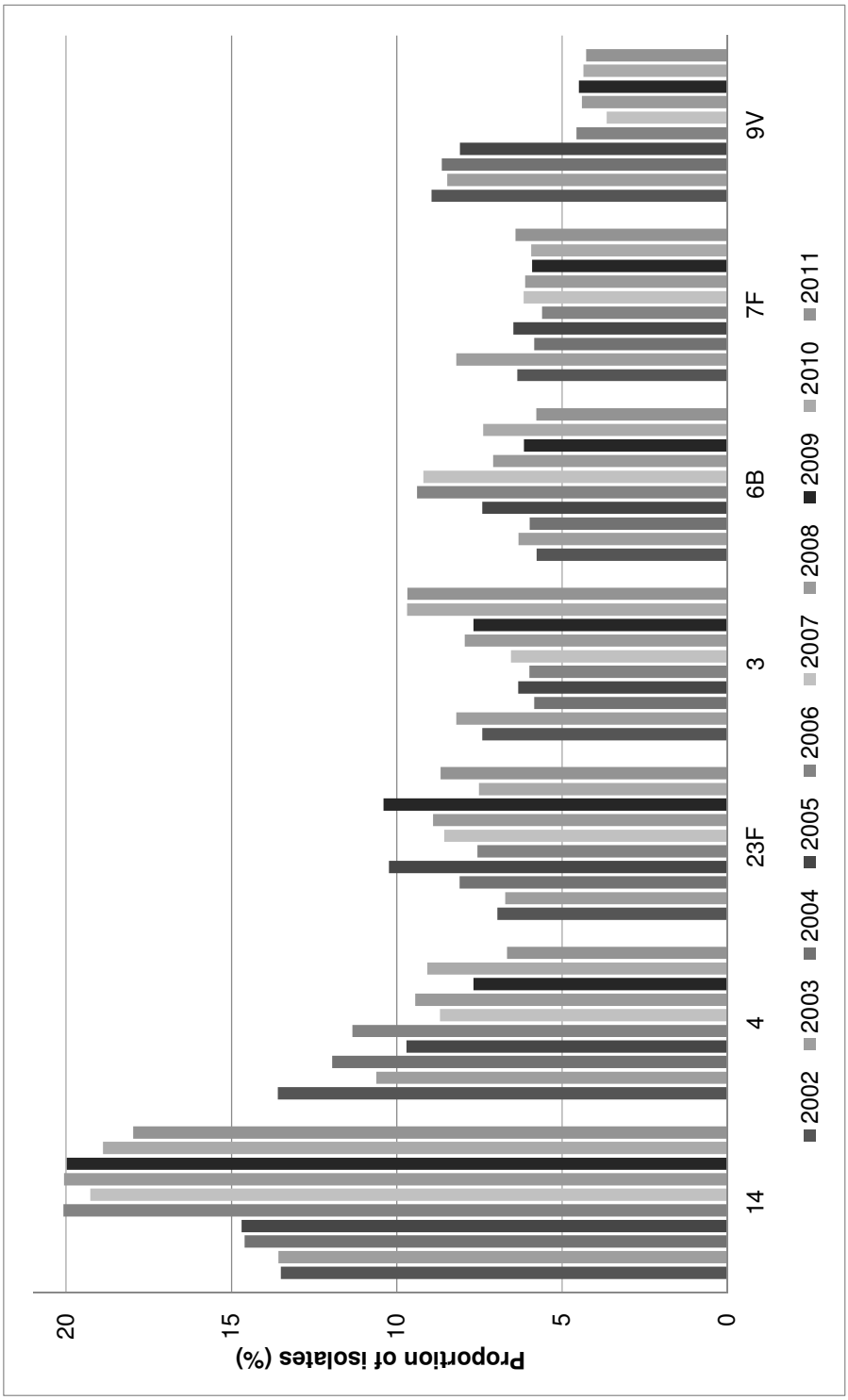

Figure 6A. The proportion of the most frequently encountered serotypes, serotypes 14, 4, 23F, 3, 6B, 7F, and 9V, among the Finnish invasive pneumococci 2002 to 2011.

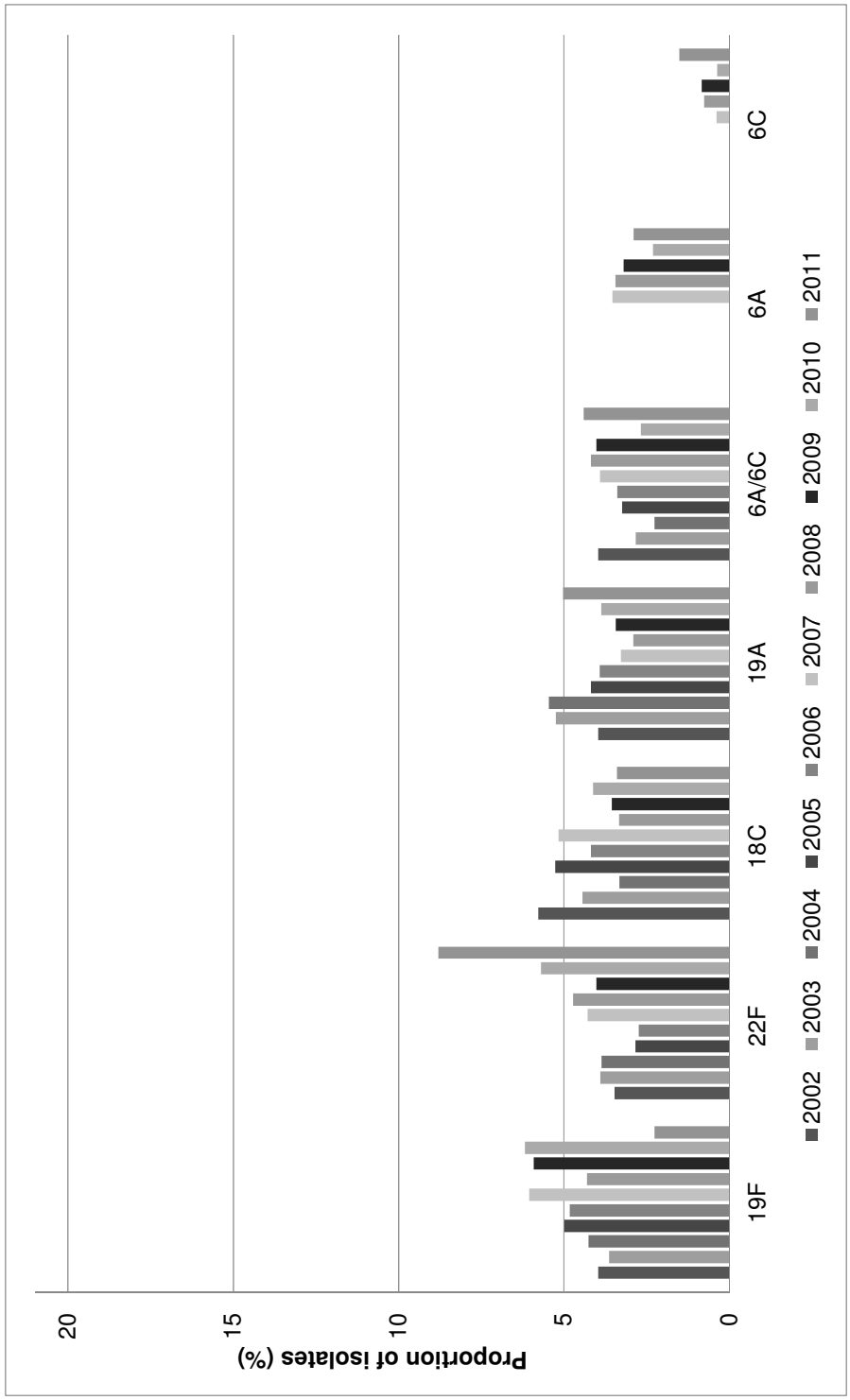

Figure 6B. The proportion of serotypes 19F, 22F, 18C, 19A, and 6A/C among the Finnish invasive pneumococci 2002 to 2011. The combined proportion of serotypes 6A and 6C are shown for the whole ten-year period, as well as separately for the latter five years.

In the first five years for the study period, the hypothetical PCV7 serotype coverage was highest at 72% among the isolates derived from 0-2-year-olds and lowest at 54% among those derived from 16-64-year-olds. The overall hypothetical serotype coverage for all age-groups and isolates was 58%. The hypothetical PCV10 coverage was 81% of the isolates obtained from 0-2 year olds in 2007, and 72% in 2011. The hypothetical PCV13 coverage in this age group was 84% in 2007, and 88% in 2011. The hypothetical PCV13 coverage overall was 68% in 2007, and 73% in 2011.

5.2 Antibiotic resistance among invasive pneumococci, 2002-2011 (I, IV)

5.2.1 Penicillin

Over the ten year study period, the proportion of penicillin-non-susceptible (MIC ≥0.12 mg/L) isolates among the invasive pneumococci increased from 8.0% (48/547) to 21.8% (173/794) (RR, 1.12; 95%CI, 1.08-1.15; *P*<0.001), range 8.0% to 23.3% (Figure 7).

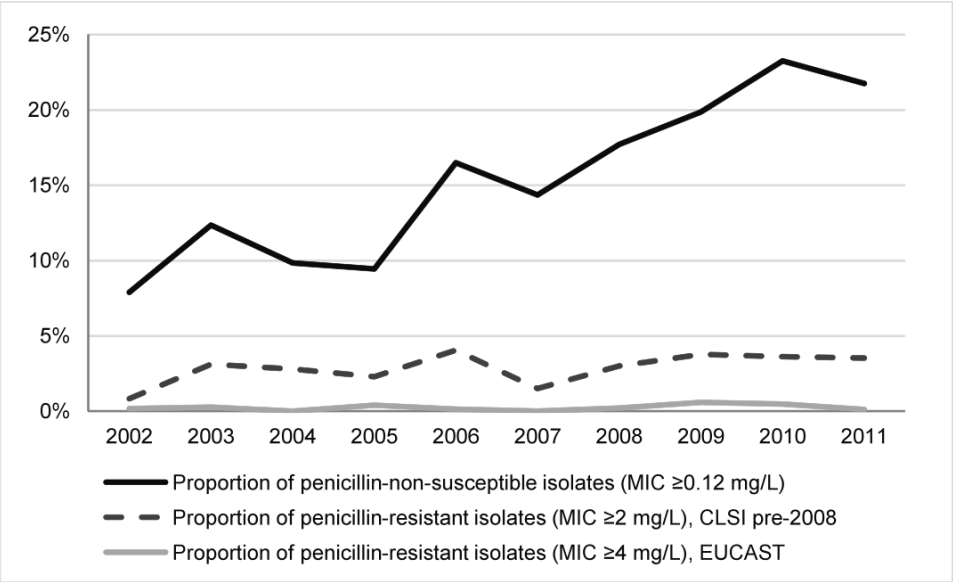

Figure 7. The proportion of penicillin-non-susceptible and -resistant isolates among invasive pneumococci isolated in 2002 to 2011.

The proportion of penicillin-resistant isolates according to the pre-2008 breakpoint issued by CLSI went from 0.8% (5/595) in 2002 to 3.5% (28/794) in 2011, range 0.8% to 3.8%, but the increase was not statistically significant. The penicillin-resistance proportion as defined by EUCAST also remained stable at 0.2% overall, range 0% to 0.6% (Figure 7). Only two isolates were found that were resistant to penicillin as defined by the 2008 CLSI breakpoint; these were isolated in 2010 and 2011, respectively.

When the five year study periods were examined separately, the proportion of penicillin-non-susceptible isolates (MIC ≥0.12 mg/L) doubled from 8.0% to 16.4% (RR, 1.15; 95%CI, 1.07-1.23; $P<0.0001$) and the proportion of penicillin-resistant isolates, as defined by the pre-2008 CLSI breakpoint, increased from 0.8% to 3.7% (RR, 1.18; 95%CI, 1.02-1.36; $P=0.03$). In the latter five years, the proportion of penicillin-non-susceptible isolates increased from 14.4% to 21.8% (RR, 1.11; 95%CI, 1.05-1.17; $P<0.001$, but the penicillin-resistance did not change significantly.

The proportion of penicillin-non-susceptible isolates was high within some serotypes (Figure 8), and in four serotypes it increased significantly over the ten-year period. The proportion of penicillin-non-susceptible isolates among serotype 14 increased from 25.0% in 2002 to 70.6% in 2011 (RR, 1.11; 95%CI, 1.08-1.15; $P<0.001$); among serotype 19A from 12.5% to 37.5% (RR, 1.13; 95%CI, 1.02-1.26; $P=0.024$); among serotype 4 from 1.2% to 26.4% (RR, 1.41; 95%CI, 1.28-1.54; $P<0.001$); among serotypes 6A and 6C combined from none to 11.4% (RR, 1.93; 95%CI, 1.42-2.61; $P<0.001$).

Over the ten year period, the proportion of penicillin-non-susceptible isolates increased in all age-groups. Among the 0-2-year-olds it increased from 15.7% (11/70) in 2002 to 29.7% (19/64) in 2011 (RR, 1.15; 95%CI, 1.08-1.21; $P<0.001$), among the 3-15-year-olds from 6.3% (2/32) to 31.0% (9/29) (RR, 1.18; 95%CI, 1.10-1.26; $P<0.001$), among the 16-64-year-olds from 8.7% (28/324) to 20.8% (84/403) (RR, 1.12; 95%CI, 1.08-1.16; $P<0.001$), and among the ≥65-year-olds from 5.7% (10/179) to 20.4% (61/299) (RR, 1.07; 95%CI, 1.01-1.13; $P=0.034$).

When studied separately in five year periods, the proportion of penicillin-non-susceptible isolates increased only among the 16-64 year-olds (RR, 1.22; $P<0.001$) and among the ≥65-year-olds (RR, 1.15; $P=0.02$) in 2002 to 2006. In 2007 to 2011, the increase in the proportion of penicillin-non-susceptible isolates was not significant in the youngest age-group, however, there was significant increase in all other age-groups: among the 3-15-year-olds (RR, 1.23; 95%CI, 1.13-1.34; $P<0.001$),

the 16-64-year-olds (RR 1.11; 95%CI, 1.05-1.19; *P*=0.001), among the ≥65-year-olds (RR 1.09; 95%CI, 1.03-1.14; *P*=0.002).

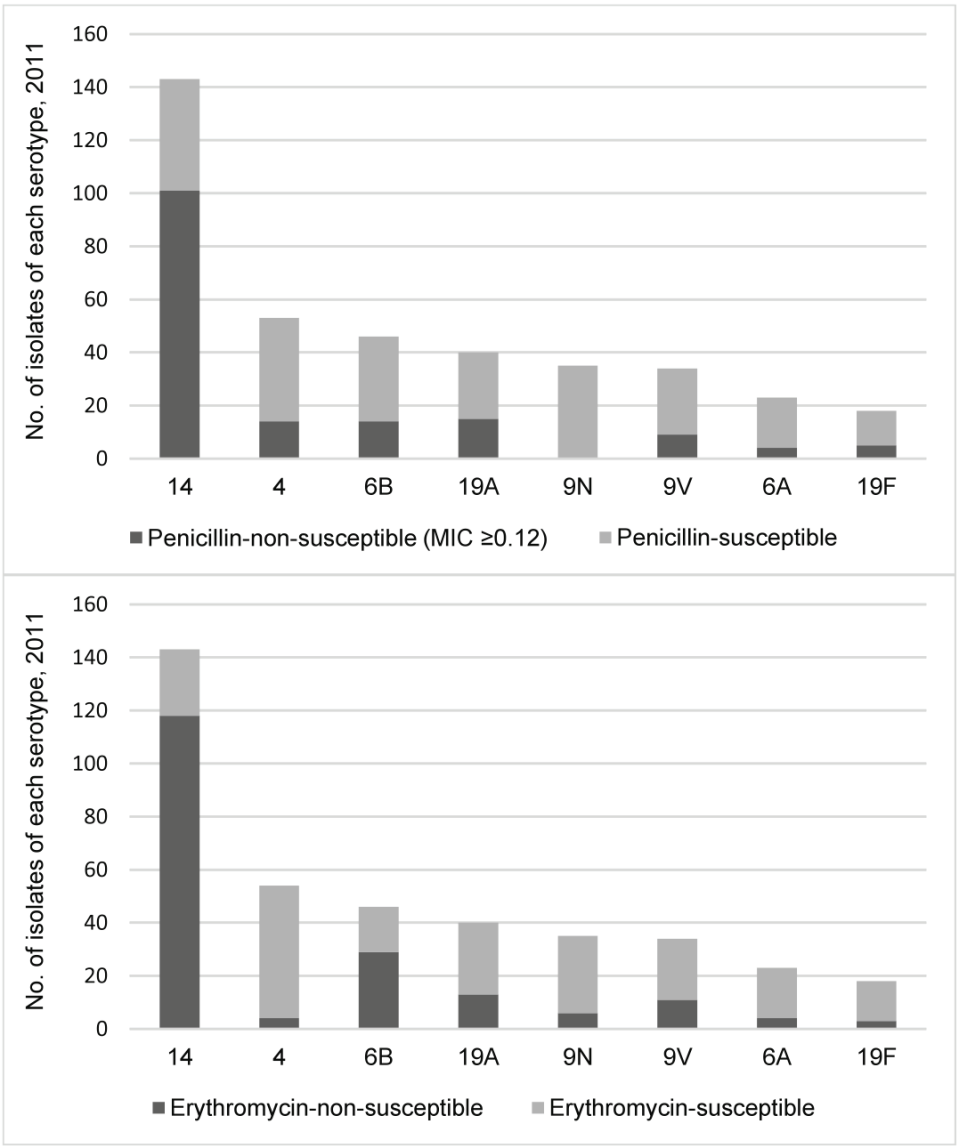

Figure 8. Penicillin non-susceptibility in selected serotypes among the invasive pneumococci isolated in 2011, top panel. Erythromycin non-susceptibility in selected serotypes among the invasive pneumococci isolated in 2011, bottom panel.

The proportion of penicillin-non-susceptible isolates (MIC ≥0.12 mg/L) in the year 2011 varied between 18.2% and 25.6% in the five tertiary care districts. In the first

five years of the study, the proportion of penicillin-non-susceptible isolates increased significantly in the tertiary care districts of Tampere, from 5% in 2002 to 15% in 2006 (RR, 1.25; 1.07-1.45; *P*=0.004), and Kuopio, from 5% to 20% (RR, 1.30; 1.04-1.61, *P*=0.02). The latter five years, the increase was significant in the districts of Helsinki from 18% to 26% (RR, 1.10; 95% CI, 1.01-1.20; *P*=0.033), Kuopio from 8% to 23% (RR, 1.30; 95% CI, 1.27-1.33; P<0.001), and Turku from 11% to 19% (RR, 1.10; 95% CI, 1.00-1.22; P=0.044).

In the first five years of the study, the hypothetical PCV7 coverage was 87% of the penicillin-non-susceptible isolates; in the last five years, the hypothetical PCV10 coverage was 90% of the penicillin-non-susceptible isolates.

5.2.2 Other antimicrobial agents

The proportion of erythromycin-non-susceptible isolates increased over the ten-year period from 16.3% to 26.4% (RR, 1.05; 95%CI, 1.02-1.07; *P*<0.001), and was 24.3% overall, range 16.3% to 28.9% (Figure 9). In the first five years, the increase was significant from 16.3% to 28.4% (RR, 1.12; 95%CI, 1.06-1.18; *P*<0.001), however in the latter five years the proportion did not increase significantly and was 26.6% overall, range 23.2% to 28.9%.

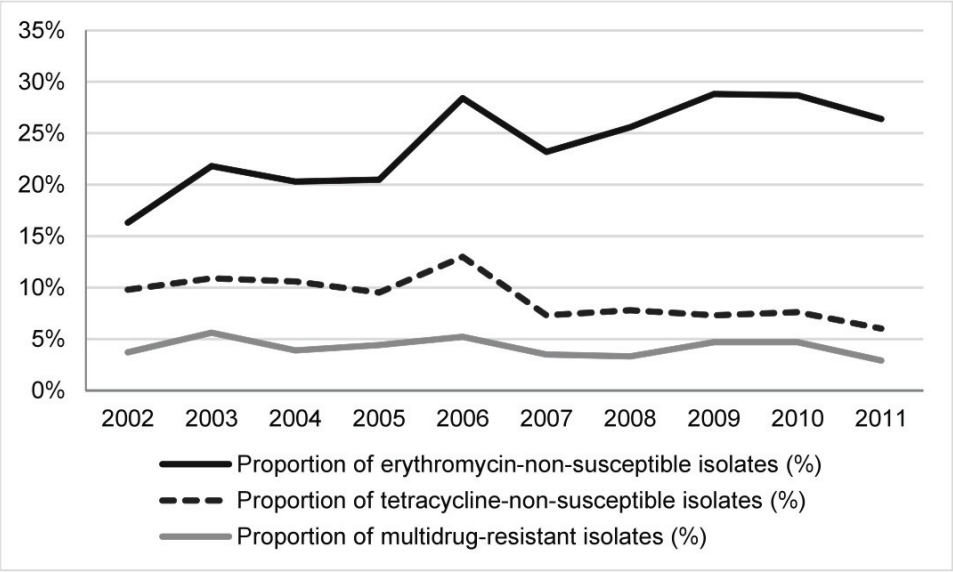

Figure 9. The proportion of invasive isolates non-susceptible to erythromycin and tetracycline, respectively, and the proportion of multidrug-resistant (MDR) isolates non-susceptible to PEN, ERY, and TET among the invasive pneumococci in Finland in 2002 to 2011.

The proportion of erythromycin-non-susceptible isolates was consistently highest in the youngest age-group. In the year 2011, the proportion of non-susceptible isolates was 46.9% among isolates from 0-2-year-olds, 27.6% among isolates from 3-15-year-olds, 25.6% among isolates from 16-64-year-olds, and 23.1% among isolates from ≥65-year-olds. The proportion of non-susceptible isolates increased among the isolates from all tested age-groups during the first five-year period, 2002 to 2006: among the 0-15-year-olds (RR, 1.12; P=0.01); among the 16-64-year-olds (RR, 1.11; P=0.003); and among the ≥65-year-olds (RR, 1.16; P<0.001). In the latter five years, there was a significant increase in non-susceptibility over the study period only among the 16-64-year-olds (RR, 1.06; P=0.049).

In the year 2011, the proportion of erythromycin-non-susceptible isolates varied between 18.7% and 34.3% among the five tertiary care districts. In 2002 to 2006, a significant increase in the proportion of erythromycin-non-susceptible isolates was observed in the tertiary care district of Tampere from 7% to 29% (RR, 1.35; 95%CI 1.22-1.51; P<0.001) and Oulu from 20% to 28% (RR, 1.12; 95% CI 1.00-1.25; P=0.04). In 2007 to 2011, the non-susceptibility increased significantly only in the district of Kuopio from 22% to 29% (RR, 1.07; 95% CI, 1.04-1.10; P<0.001).

In the first five years of the study, the hypothetical PCV7 coverage of the erythromycin-non-susceptible isolates was 80%, and in the last five years, the hypothetical PCV10 coverage was 86% of the erythromycin-non-susceptible isolates. Serotype 14 especially, but also certain other serotypes, contained a high proportion of erythromycin-non-susceptible isolates in the year 2011 (Figure 8).

The proportion of tetracycline-non-susceptible isolates decreased slightly over the ten years from 9.8% to 6.0% (RR, 0.94; 95%CI, 0.91-0.98; P=0.001) and was 8.9% overall, range 6.0% to 13.0% (Figure 9). When studied separately, there was no significant change during either of the two five year periods.

Ceftriaxone susceptibility was studied from 2005 onwards and 3.7%, range by year, 2.5% to 4.2%, of all tested isolates were non-susceptible to this antimicrobial agent. Twelve isolates or 0.2% had ceftriaxone MICs ≥2 mg/L. The non-susceptibility to levofloxacin was similarly rare at 0.2% overall, with 16 non-susceptible isolates discovered since testing started in August, 2004.

The proportion of multidrug-resistant isolates, defined as non-susceptibility to penicillin, erythromycin, and tetracycline, was 4.2% overall, range 3.3% to 5.6%, and did not change significantly over the ten years or in the two five year periods (Figure 9). Of the erythromycin-non-susceptible isolates, 38% (2002-2006) and 55% (2007-2011), respectively, were also non-susceptible to penicillin (MIC ≥0.12 mg/L).

Overall, 74% (2002-2006) and 76% (2007-2011), respectively, of the penicillin-non-susceptible isolates were also non-susceptible to erythromycin.

5.3 Macrolide resistance determinants among the invasive pneumococci, 2002-2006 (I)

The *mef* gene was the most common macrolide resistance determinant. It was carried by 56% of the 223 randomly selected erythromycin-non-susceptible isolates. Of the subtyped *mef* positive isolates, 72% carried *mef*(E) and 28% carried *mef*(A). All *mef*(A) positive isolates were serotype 14 and susceptible to penicillin, which suggests they may be clonally related. The *erm*(B) gene was present in 30% of the screened isolates. Double carriage of macrolide resistance determinants, *mef*(E) and *erm*(B), was displayed by only two of the studied isolates (0.9%). These isolates were both serotype 19F and part of CC320. The macrolide resistance mechanism remained unknown in 13% of the studied isolates.

5.4 Genotype clonality of penicillin-resistant and -non-susceptible invasive pneumococci, 2002-2011 (I, IV)

Among the penicillin-resistant (MIC ≥2 mg/L) invasive isolates from 2002 to 2006, 25 STs belonging to nine CCs and one singleton was discovered. The CCs and their STs were CC156 with ST156, ST2918, ST2306, ST3247, ST143, ST2916, ST671, ST90, ST3246, and ST138; CC320 with ST236, ST271, ST2694, ST2917, and ST3245; CC81 with ST81, ST961, and ST3250; CC15 with ST13; CC63 with ST2687; CC496 with ST496; CC205 with ST205; CC460 with ST461; and CC2599 with ST3248. The singleton was ST3249.

The penicillin-resistant (MIC ≥4 mg/L) isolates from 2007 to 2011 displayed eight STs belonging to two CCs, CC156 and CC320. These CCs were present also among the highly penicillin-resistant invasive isolates in the previous five-year period (Table 6).

The penicillin-non-susceptible isolates (MIC 0.12-1.0 mg/L) from the year 2005 displayed 34 different STs belonging to 14 CCs and one singleton (Table 7).

Among all the invasive penicillin-non-susceptible isolates genotyped by MLST, 14 novel STs were discovered. Among these, four novel *ddl* alleles, and one novel allele at the *aroE*, *gki*, *recP*, and *xpt* loci each were discovered. CC320 and lineage termed i within CC156, as defined by the eBURST algorithm are shown in Figure 10 and Figure 11, respectively.

Table 6. Clonal complexes, sequence types, serotypes, and antimicrobial susceptibility of the penicillin-resistant (MIC ≥ 4 mg/L) pneumococci isolated in the years 2002 to 2011. One isolate per case is included (*n*=20).

CC	ST	Allelic profile	Serotype	Antimicrobial susceptibility (mg/L)			Related PMEN clone	Pilus islets*	Year of isolation (no. of isolates)
				PEN	ERY	TET			
320	236	15-16-19-15-6-20-26	19F	4	8	64	PMEN14 Taiwan^{19F}ST236	PI-1	2006 (1)
	2917	4-16-19-15-6-20-266	19F	4	>128	32		PI-1, PI-2	2005 (1), 2009 (2)
	320	4-16-19-15-6-20-1	19F	4	>128	0.25		PI-1, PI-2	2010 (1)
	320	4-16-19-15-6-20-1	19A	8	>128	64		PI-1, PI-2	2011 (1)
	271	4-16-19-15-6-20-26	19F	4-8	>128	16-32		PI-1, PI-2	2009 (2), 2010 (1)
	3245	4-16-19-15-6-20-286	19F	4	>128	32		PI-1	2002 (1)
	651	15-5-19-15-6-20-26	19F	4	4	0.25		PI-1, PI-2	2009 (1)
	8794	15-16-19-234-6-20-50	19F	4	>128	32		PI-1, PI-2	2010 (1)
156	156	7-11-10-1-6-8-1	14	4	0.125	0.25	PMEN3 Spain^{9V}ST156	PI-1	2005 (1)
	143	7-5-10-18-6-8-1	14	4	>128	0.25		PI-1	2008 (1)
	2916	7-5-10-18-6-5-1	14	4	>128	64		PI-1	2008 (1)
	2918	7-11-10-1-6-8-267	14	4	>128	0.5		PI-1	2010 (1)
	138	7-5-8-5-10-6-14	6B	4	16	0.25		PI-1	2006 (1)
81	81	4-4-2-4-4-1-1	23F	4	0.125	1	PMEN1 Spain^{23F} ST81	None	2003 (1)
15	13	1-5-4-5-5-27-8	14	4	32	0.25	PMEN9 England^{14}ST9	None	2003 (1)
63	2678	2-60-36-12-17-21-14	14	4	>128	64	PMEN25 Sweden^{15A}ST63	None	2005 (1)

CC, clonal complex; ST, sequence type; PEN, penicillin, ERY, erythromycin; TET, tetracycline; PMEN, Pneumococcal Molecular Epidemiology Network; PI-1, pilus islet 1; PI-2, pilus islet 2. *PI-1 was detected in the isolates from 2002-2006, while both PI-1 and PI-2 were detected in the isolates from 2007-2011.

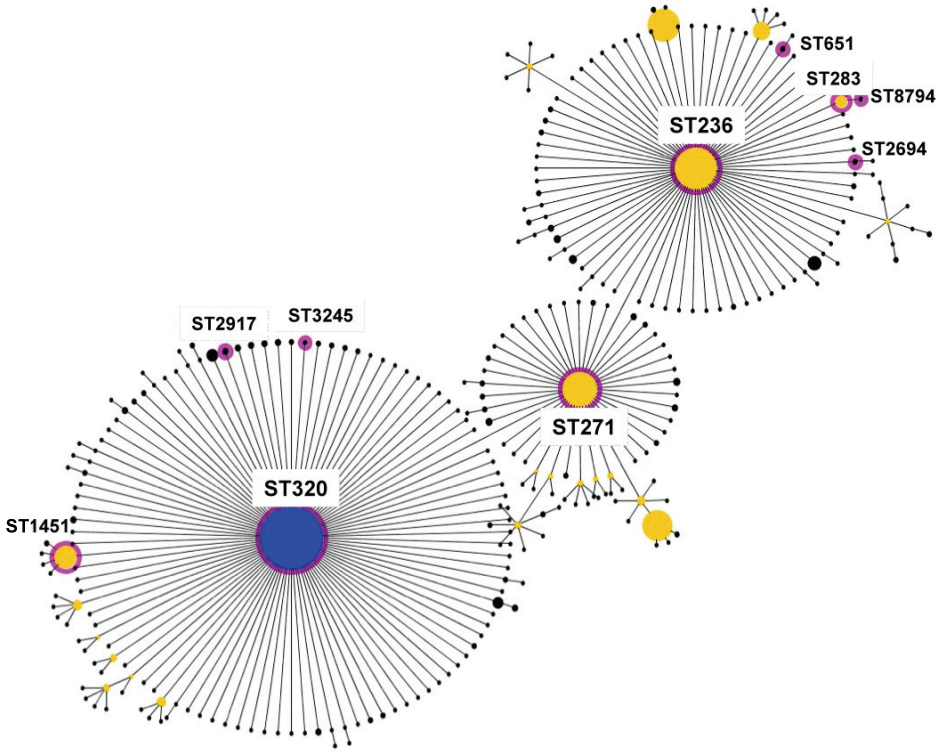

Figure 10. CC320 as defined by the eBURST algorithm [75]. The predicted CC founder ST320 is depicted in blue, subgroup founders in yellow. The magenta halos depict isolates found in the material in this study, all CC320 STs from both the invasive and the non-invasive isolates in Publications I, II, IV and Unpublished, Siira *et al.* are included.

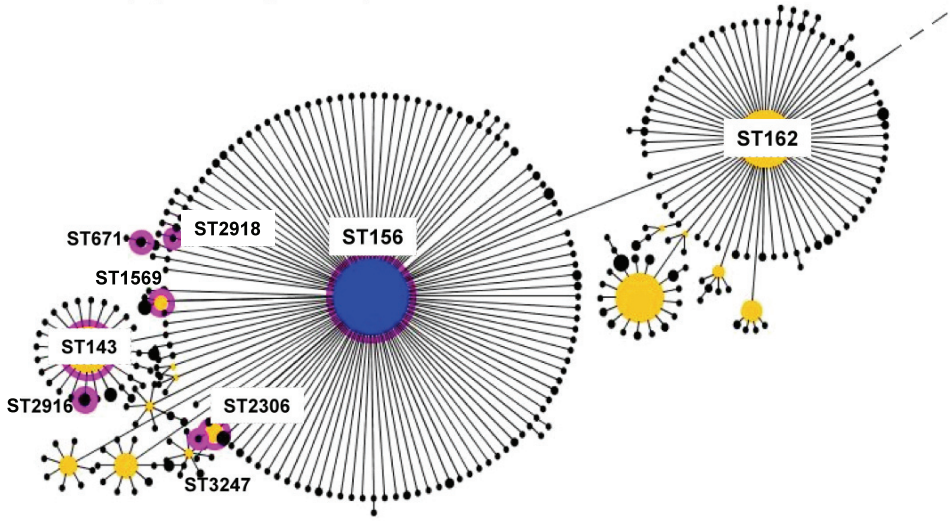

Figure 11. The genetic lineage i within CC156 as defined by the eBURST algorithm and the 96-MLST scheme [75, 221]. The magenta halos depict isolates found in the material in this study, the CC156 STs from both the invasive and the non-invasive isolates in Publications I, II, IV and Unpublished, Siira *et al.* are included. The complete CC156 is depicted in Figure 4.

Table 7. Clonal complexes, sequence types, serotypes, and antimicrobial susceptibility of the penicillin-non-susceptible pneumococci isolated in the year 2005. One isolate per case is included (*n*=66).

CC	ST	Allelic profile	Sero-type	Antimicrobial susceptibility (mg/L)			No. of isolates	Related PMEN clone
				PEN	ERY	TET		
156	156	7-11-10-1-6-8-1	9V	2	0.125	0.25	1	PMEN3 Spain^{9V}ST156
	156	7-11-10-1-6-8-1	14	1-4	0.063-16	0.25-0.5	5	
	156	7-11-10-1-6-8-1	9N	1	0.063-0.125	0.25	2	
	143	7-5-10-18-6-8-1	14	2	>128	32	1	
	671	7-11-10-1-5-76-98	14	0.125	32-64	0.125-0.5	9	
	2916	7-5-10-18-6-5-1	14	1-2	>128	32	2	
	2306	7-11-10-1-6-8-119	14	2	>128	32	1	
	1569	8-11-10-1-6-8-1	9V	1	>128	64	1	
	338	7-13-8-6-1-6-8	23F	0.125	0.125	0.25	1	PMEN26 Colombia^{23F}ST338
	90	5-6-1-2-6-3-4	6B	1-2	>128	0.5-64	3	PMEN2 Spain^{6B}ST90
	9069	7-5-8-5-10-6-570	6B	0.125	0.125	0.25	1	
63	63	2-5-36-12-17-21-14	14	0.25	0.125	0.25	1	PMEN25 Sweden^{15A}ST63
	782	2-5-36-12-17-21-5	14	0.125-0.25	>128	32-64	6	
	2678	2-60-36-12-17-21-14	14	1-4	>128	32-64	3	
315	315	20-28-1-1-15-14-14	6B	0.125	>128	32-64	2	PMEN20 Poland^{6B}ST315
	386	32-28-1-1-15-52-14	6B	0.125	>128	32	4	
	9068	20-28-1-1-15-526-14	6B	0.125	64	0.5	1	
230	319	12-19-2-17-6-22-9	19F	0.5	>128	32	3	PMEN32 Denmark^{14}ST230
	276	2-19-2-17-6-22-14	19A	1	>128	0.5	1	
	2562	11-19-2-17-6-215-75	19F	0.5	>128	32	1	
	9108	12-19-70-17-6-22-14	19A	0.25	0.125	32	1	

CC	ST	Allelic profile	Sero-type	Antimicrobial susceptibility (mg/L)			No. of isolates	Related PMEN clone
				PEN	ERY	TET		
320	271	4-16-19-15-6-20-26	19F	2	>128	32	1	PMEN14 Taiwan^{19F}ST236
	2694	15-16-19-1-6-20-26	19F	1	4	1	1	
	2917	4-16-19-15-6-20-266	19F	2-4	>128	32	2	
15	15	1-5-4-5-5-3-8	14	1	>128	0.25	1	PMEN9 England^{14}ST9
	13	1-5-4-5-5-27-8	14	2	16	0.25	1	
2660	2660	2-2-100-5-15-3-8	6A	0.125	0.031	32	1	
	9109	2-2-100-5-15-3-14	6A	0.25	0.063	32	1	
81	81	4-4-2-4-4-1-1	23F	2	2	0.25	1	PMEN1 Spain^{23F} ST81
180	180	7-15-2-10-6-1-22	3	0.125	>128	0.5	1	PMEN31 Netherlands^{3}ST180
199	199	8-13-14-4-17-4-14	19A	0.125	16	0.5	1	PMEN37 Netherlands^{15B}ST199
346	3557	7-41-47-16-6-14-17	19F	0.5	0.125	0.25	1	
439	1682	1-8-9-1-6-4-175	23F	0.125	0.125	0.25	1	
496	496	42-35-29-36-9-39-18	18C	0.125	0.5	0.25	1	
1379	1292	1-13-9-12-94-28-20	23F	1	4	0.25	1	
Singleton	6864	118-60-4-15-25-14-9	4	0.125	0.125	0.25	1	

CC, clonal complex; ST, sequence type; PEN, penicillin, ERY, erythromycin; TET, tetracycline; PMEN, Pneumococcal Molecular Epidemiology Network.

5.5 Pilus islets among the penicillin-resistant invasive pneumococci, 2002-2011 (I, IV)

Pilus islet carriage was limited to STs of certain CCs. Among the penicillin-resistant pneumococci (MIC ≥2 mg/L) isolated from 2002 to 2006, PI-1 was carried by the isolates in CC156, CC320, CC205 and the singleton ST3249. CC81, CC460, CC2599, CC15, CC63 and CC496 were negative for PI-1. The isolates isolated before 2007 were not studied for the presence of PI-2. Of the penicillin-resistant (MIC ≥2 mg/L) isolates from the years 2002 to 2006, 79.5% carried PI-1 if extrapolated from the results of the studied ST representatives. In 2007 to 2011, all penicillin-resistant isolates (MIC ≥4 mg/L) carried PI-1, and in addition, the serogroup 19 CC320 isolates (75%) also carried PI-2 (Table 6).

5.6 Characteristics of non-invasive multidrug-resistant pneumococci (II)

Five serotypes were found among the 12 isolates: 19F (*n*=5), 19A (*n*=3), 6B (*n*=2), 23F (*n*=1), and 14 (*n*=1). Six STs from three genetic CCs were represented: CC320 with ST271 (*n*=4), ST1451 (*n*=3), ST283 (*n*=1); CC156 with ST90 (*n*=2), ST2918 (*n*=1); and CC81 with ST81 (*n*=1). CC320 claimed all serogroup 19 isolates. CC320 and the lineage termed i within CC156, as defined by the eBURST algorithm, are depicted in Figure 10 and Figure 11, respectively. Of the 12 isolates, 11 (92%) carried PI-1 and 8 (67%) carried PI-2. All CC320 isolates were positive for both pilus islets, while the CC156 isolates were positive only for PI-1. The CC81 isolate was negative for both pilus islets. PI-2 was associated with serogroup 19 and with CC320 (P=0.002). All CC320 isolates displayed the double macrolide resistance determinant phenotype, carrying both *erm*(B) and *mef*(A/E), while the CC156 and CC81 isolates carried only the *erm*(B) gene.

5.7 Validation of the mPCR based serotyping scheme (III)

Overall, the three sets consisted of 42 different serotypes and three non-encapsulated pneumococci and one non-pneumococcal isolate (Figure 12). The initial results obtained at the two THL laboratories by mPCR-based serotyping supplemented with Quellung (Figure 13) in Helsinki and by traditional immunological methods in Oulu, were compatible for 95.3% (162/170) of the isolates overall. For the first validation set, the results from the THL laboratories in Helsinki and Oulu were congruent for 97% (68/70) of the isolates; for the second set, for 96% (67/70) of the isolates; and for the third set, for 90% (27/30) of the isolates. For the serotypes where the protocols used differed in resolution power, results such as serotype 10F/C vs. serogroup 10 were not considered contradictory.

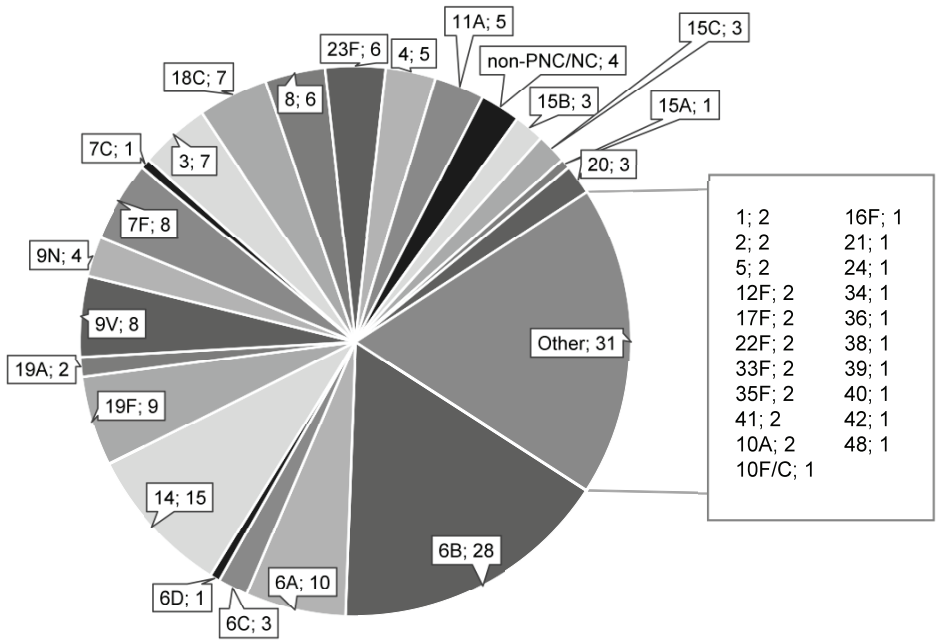

Figure 12. Serotypes included in the three validation sets (serotype; number of isolates) sorted by serogroup. Serotypes are named to the level that is in use for the mPCR based serotyping scheme for invasive pneumococci in Finland. PNC, pneumococcus; NC, non-encapsulated.

External verification of discrepant serotyping results was performed at one or both of the reference laboratories at SSI and UAB. Following external verification, the results obtained by the mPCR-based serotyping scheme were compatible with the verified results for 98.6% (69/70) of the first validation set, 98.6% (69/70) for the second set, and 100% (30/30) for the third set. The combined compatibility between the results obtained by the mPCR based serotyping scheme and the external reference laboratory results was 98.8% (168/170). For isolate IH163205-2, serotype 6D, the serotyping performed at UAB was considered decisive, as this was where serotype 6D was first described and studied in detail [34, 35]. The results for this isolate have been published separately [229]. For isolate IH163216-4, the result of non-pneumococcus from SSI was considered decisive, as species verification tests had been performed there.

Together, the first two mPCRs were anticipated to give a result for over 80% of the invasive isolates when setting up the new serotyping scheme (Figure 13). This expectation proved accurate, as 86% of the invasive pneumococci isolated in the initial 18 months of application produced either a serotype or serogroup specific amplicon in one of the first two mPCRs. Routine application of the serotyping scheme was successful and interpretation of the electrophoresis gel amplicon patterns was straightforward. It also confirmed that the six main mPCRs combined produced a serotype- or serogroup-specific mPCR amplicon for 99.4% of the Finnish invasive isolates.

For serotyping validation set 1, the approximate mean material cost by Quellung was 17 € per isolate, range 14-20 €. The approximate mean material cost for serotyping by the mPCR based serotyping scheme including Quellung when needed, as depicted in Figure 13, was 6 € per isolate, range 3-13 €.

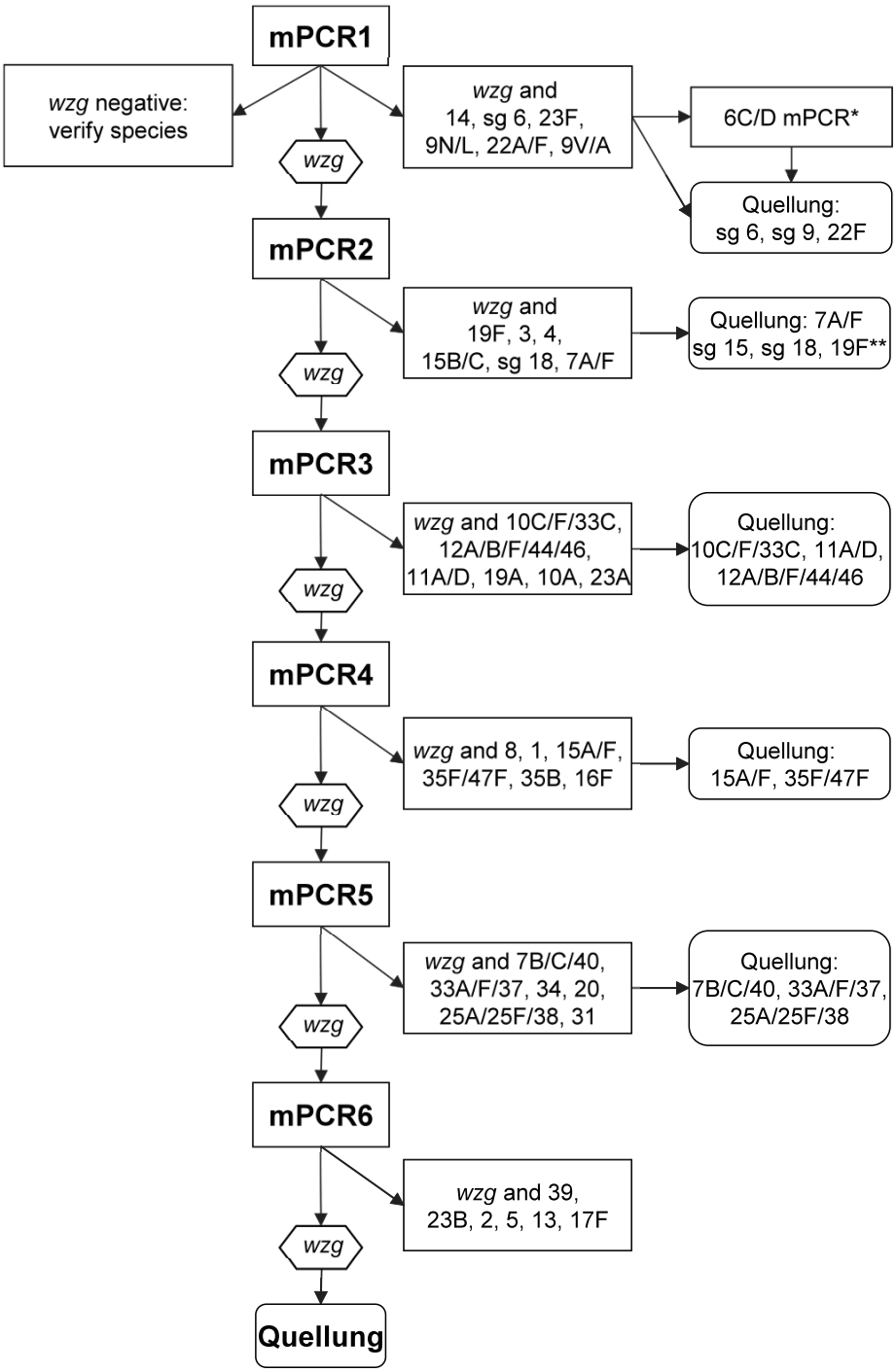

Figure 13. Flowchart for multiplex PCR based serotype deduction supplemented with Quellung. The *wzg* amplicon is the positive internal control. *6C/D mPCR has not routinely been used since mid-2010; ** since late 2010 all mPCR 19F-positive isolates have been confirmed by Quellung subtyping.

5.8 The genotypes and *wzy* sequences of two serotype 19F-like 19A isolates

Two isolates that produced the 19F specific amplicon in mPCR 2, but were serotype 19A when examined by Quellung, were studied by MLST. These isolates had novel STs. Isolate IH163643-0 had ST8025 and had the allelic profile 1-5-41-5-9-1-18, while isolate IH166473-6 had ST8026 and had the allelic profile 13-8-4-5-9-1-18.

The sequenced *wzy* genes were identical in both isolates and showed 93-92% identity with the serotype 19F *wzy* genes and 80% identity with the serotype 19A *wzy* genes from other isolates found in GenBank. The *wzy* gene sequences of isolates IH163643-0 and IH166473-6 were deposited in GenBank under the accession numbers JX112900 and JX112901, respectively.

6 Discussion

6.1 Serotypes among the invasive pneumococci

The proportions of individual serotypes may fluctuate over time in a pneumococcal population even prior to extensive use of vaccines or antimicrobials. The use of antimicrobials is likely to have affected the serotype distribution before systematic surveillance of resistance was implemented [99, 122]. In this study, the three most frequently identified serotypes in the years 2002 and 2007 were all included in PCV10. The serotypes were 4, 14, and 9V in 2002; and 14, 6B, and 4 in 2007. In contrast, in 2011, the three most frequent serotypes were 14, 3, and 22F, and the proportion of all these serotypes increased over the study period. Serotype 14 covered 17.5% of the isolates from the whole period, while it only covered 12.2% of invasive pneumococcal disease cases in 1995 to 2002 [163]. The increase of serotype 22F, especially, was quite dramatic in the last year of the study reaching 8.8% in 2011. In 1995 to 2002 serotype 22F covered only 3.5% of the isolates [163]. Of the three most common serotypes isolated in 2011, only serotype 14 is a vaccine serotype, while serotypes 3 and 22F may turn out to be important in replacement following PCV10 vaccination. Serotype replacement by serotype 3 and 22F has been described elsewhere following PCV7 use [212, 249]. In this study, the proportions of the PCV10 serotypes 4 and 9V declined over the study period of ten years, 2002-2011. In the latter half of the study period, the PCV10 serotypes combined also decreased, although they had remained stable in the previous five years. These changes may not be a direct consequence of vaccine use, but their timing is interesting in relation to the PCV10 implementation in Finland in September 2010, and they warrant careful monitoring in the coming years. It has been proposed that replacement serotypes will include serotypes which are metabolically less costly for the bacteria to produce and therefore may be more heavily capsulated [329]. These low carbon containing serotypes include serotype 3, while other potential replacement serotypes proposed by this theory are 19A and 6A.

The hypothetical serotype coverage calculated based on the serotypes discovered in this material may not give the complete picture. For PCV13, the immunological response to serotype 3 has been low in some studies, for example [233]. This is an important consideration to bear in mind when comparing the serotype coverage provided by different PCVs, especially as the proportion of serotype 3 has been increasing in Finland, possibly partly in response to the use of PCV10. The distribution of invasive serotypes may also differ markedly for example from the distribution of serotypes in non-bacteraemic pneumococcal pneumonia. This was

illustrated by a recent Danish study, in which the PCV10 and PCV13 serotypes covered only a sixth and a third of non-invasive cases, respectively, while the proportions were much higher for the bacteraemic cases [22]. In a study from the UK, the loss of quality of life, clinical presentation, and mortality was calculated and found to differ markedly between serotypes, giving different vaccines different disease coverage profiles [315].

The proportions of serotype 14 and serotype 9V changed radically and simultaneously from one year to the next during the study. These changes took place from 2005 to 2006, when serotype 14 increased, while serotype 9V decreased. It is tempting to speculate that this may have been caused by the rise of a particularly successful serotype 14 clone at the expense of a declining serotype 9V clone.

Interestingly, serotype 3, has in some studies been found to display low invasiveness [38], but was frequently encountered in invasive samples in Finland during the whole ten year period. Other studies have found that this serotype has a high invasive disease potential especially among adults and the elderly, and also high mortality [249, 285, 339]. On the other hand, serotype 1, which has high invasive disease potential [38], is very rare among the invasive isolates in Finland. This may perhaps be explained by the low number of strains of this serotype circulating in human society. Similarly low serotype 1 proportions have been described in the USA, Canada, and Australia, while in much of Europe, Asia, Latin America and Africa higher proportions of up to 30% are found [127].

6.2 Recently discovered and aberrant serotypes

6.2.1 Serogroup 6

In the years covered by this study, several novel serotypes have been discovered. This is most notable within serogroup 6, where the two previously known serotypes, 6A and 6B, have been joined by serotypes 6C and 6D, and even more recently, by the proposed serotypes 6E, 6F, and 6G [34, 164, 237, 244]. Serotypes 6A and 6B, the two oldest members of this serogroup, were described in the late 1920s [130]. Genetically, they differ from each other only by a single nucleotide polymorphism at the 584 bp position in the *wciP* gene, which changes the amino acid residue at position 195 [200, 292]. This is the genetic basis for the different rhamnose ribitol linkages found in the serotype 6A and 6B capsular polysaccharides: a 1,3 linkage in serotype 6A and a 1,4 linkage in serotype 6B [200].

Serotype 6A subtypes were suspected already in 2006, when “dodgy 6As” isolates were found. These bound only to certain types of monoclonal antibodies developed

against serotype 6A and displayed a weak reaction with the Pool B antiserum in the Quellung reaction [123, 182]. A year later, the genetic basis for serotype 6C was described. This serotype is similar to serotype 6A, but carries the *wci*N$_{\beta}$ gene, a roughly 200 bp shorter version of the serotype 6A *wci*N$_{\alpha}$ gene. The gene product is a α-1,3-galactosyltransferase involved in capsular production. Pneumococci with this serotype have been isolated from both invasive and non-invasive samples from around the world [243].

A few years after the discovery of serotype 6C, the same research group generated an analogous variant of serotype 6B, i.e. an isolate that harboured the serotype 6B *wciP* gene, but also carrying the *wci*N$_{\beta}$ gene. This serotype was named 6D [35]. Isolates carrying this capsular type were later discovered among natural clinical isolates [34], and a study from South Korea has established that serotype 6D has been circulating there at least since 1996 [51]. In Finland, three serotype 6D isolates have been discovered among the invasive pneumococci since the initial publication of the serotype. The isolates are included in this study, and one of them has been described separately [229]. In addition to this, there are only a handful of reports of invasive serotype 6D isolates worldwide to date [51, 173, 199, 262, 346]. According to reports from several countries, it appears to be more commonly encountered among non-invasive isolates [151, 157, 165, 208, 236, 276].

In this study, the serotype 6A isolates from the years 2007 to 2011 were studied either by 6C/6D mPCR or the Quellung reaction to differentiate the serotype 6C isolates. This showed that serotype 6C had been present among the invasive isolates at least since the year 2007. As not all previous serotyping results have been confirmed using the larger selection of serogroup 6 specific antisera or the 6C/6D specific mPCR, it is likely that these newer serotypes may be present also among older isolates. Serotype 6C has been in existence at least since 1979 [243] and it appears that the serotype 6C *cps* has appeared through several independent capsular switching events within several different genetic lineages [187]. Because of its rarity among the invasive pneumococci isolated since 2010, there has at this time been no attempt to retrospectively distinguish serotype 6D from 6B in Finland. It is still unclear whether the other proposed serotypes of this serogroup, serotypes 6E, 6F and G, are more likely to be encountered in invasive or non-invasive sites and how common they are.

This study and others show that among the serogroup 6 serotypes, penicillin-non-susceptibility is most frequent in serotype 6B. Serotype 6A isolates display non-susceptibility (MIC ≥0.12 mg/L) in some cases, though rarely full resistance [202, 313]. Penicillin-non-susceptibility among serotype 6C is low, but has increased at least in the USA, exceeding 30% among invasive isolates in the year 2007 [46]. In Portugal, multidrug-resistance among serotype 6C has been on the increase [234]. In

the Finnish material, only one penicillin-non-susceptible serotype 6C isolate was discovered between 2007 and 2011, therefore one can conclude that the increase in non-susceptibility in serotype 6A and 6C combined is largely due to serotype 6A non-susceptibility. All three Finnish serotype 6D isolates in this study were susceptible to the tested antimicrobials, and many other studies similarly report penicillin-susceptible serotype 6D isolates [202, 313, 346]. However, in a large collection containing both invasive and non-invasive serotype 6D isolates from Israel, more than 90% displayed non-susceptibility to at least one antimicrobial agent and multidrug-resistance exceeded 70% [262]. This finding underlines the importance future monitoring, especially as non-invasive pneumococcal clones appear to have a tendency to develop resistance [137].

From a PCV standpoint, the discovery of any new serogroup 6 serotypes is of interest. Serotype 6B is included in all three available PCVs, and additionally, serotype 6A is included in PCV13. The discovery of serotype 6C raised concerns of post-vaccination replacement. Indeed, in the PCV7 era serotype 6C makes up the majority of invasive serogroup 6 isolates in the USA and has increased in Australia [46, 211, 346]. A study from the UK reported a four-fold increase in serotype 6C carriage in the PCV7 era, due to one genetic lineage, CC395 [187]. By contrast, in Germany, the prevalence of serotype 6C has remained low throughout the period of PCV7, PCV10, and PCV13 use [313]. Preliminary results concerning PCV13 indicate that it may provide cross-protection against serotypes 6C and 6D, and that PCV7 may offer cross-protection against serotype 6D [58, 59, 202]. Similarly interesting from a prevention standpoint is that the epidemiology of newly defined serotypes may differ from the previously known ones, as suggested by a South African study, which showed that serotype 6C seems more prone to cause meningitis than 6A [83].

Other newly described serotypes from other serogroups may also be present among the invasive pneumococci in Finland. Preliminary testing of a monoclonal antibody based serotyping scheme [341] indicated the presence of an 11E isolate (Unpublished, Siira *et al*.). This serotype is not discernible by either mPCR or the Quellung reaction and would currently be serotyped as 11A. Taken together, all these findings indicate that the newly described serotypes have been present in the pneumococcal population for a long time, but their clinical significance may become apparent only when the use of serotype-specific interventions, e.g. serotype-dependent vaccines, exercise selection pressure on the pneumococcal population.

6.2.2 Serogroup 19

Novel and aberrant serotypes, for which the molecular and phenotypic serotyping results are not in agreement, have been described in the advent of new serotyping

methods. In 2009, a serotype 19F isolate produced a serotype 19A specific PCR amplicon. This discovery led to development of the improved 19A specific oligonucleotide primers designed at the CDC [258] that were used in the current study. Following the implementation of the mPCR-based serotyping protocol into continuous use at THL, Helsinki, a discrepancy between the results obtained by mPCR and Quellung was discovered. The isolate in question was serotype 19A by Quellung but had produced a serotype 19F amplicon in mPCR. The isolate did not produce any amplicon with the 19A specific oligonucleotide primers. As a precaution, all isolates that produced 19F-specific mPCR results were retrospectively and subsequently subtyped by Quellung (Figure 13), and one further discrepancy was discovered. The two isolates producing discrepant serotyping results by mPCR and Quellung each had novel genotypes, ST8025 and ST8026, with four shared alleles. The closest matches for these isolates in the MLST database were serotype 19A, 19F, and 10A isolates [227].

The capsular polysaccharide synthesis loci of serotype 19A and serotype 19F are very similar. It has been proposed that differences in one gene, *wzy* (formerly *cpsI*), give rise to the two different serotypes [220]. This gene encodes the Wzy polymerase that links oligosaccharide repeat units to form lipid-linked capsular polysaccharides. Wzy determines the serotype by facilitating different types of bonds between the otherwise identical oligosaccharide repeat units in the capsules of serotypes 19A and 19F [23, 220]. The nucleotide identity between *wzy* of serotype 19A and serotype 19F is 78.5%. The amino acid identity between the two Wzy proteins is 80.7% [220, 240, 258]. The *wzy* gene in the two Finnish isolates that gave discrepant results by mPCR and Quellung showed 92-93% nucleotide identity with the serotype 19F *wzy* sequences in the GenBank database and 80% identity with the serotype 19A *wzy* sequences, which explains the amplification with the 19F-specific *wzy* primers.

As with, serogroup 6, serogroup 19 is interesting from a PCV perspective, since 19F is included in all available vaccines, but 19A is covered only by PCV13. As mentioned previously, serotype 19A is a frequently observed replacement serotype following PCV7 use [212]. Several genotypes connected with serogroup 19 have undergone capsular switching, which illustrates the plasticity of these strains [154, 227]. At least one serotype 19A vaccine escape recombinant has been identified [37]. It has been suggested that PCV7 may have a lower effectiveness against serotype 19F than against the other vaccine serotypes [184] and this serogroup warrants careful surveillance, regardless of the vaccine in use.

6.3 Validation of the mPCR based serotyping scheme

Serotype deduction based on the pneumococcal genotype has recently gained ground. Although indirect, the methods appear to be reliable and precise, especially if combined with phenotypic methods when needed. In recent years, a variety of molecular methods that rely on the *cps* locus for the deduction of pneumococcal serotypes have been described [20, 87, 167, 168, 240, 306]. The mPCR based serotyping scheme originally developed at the CDC has been modified in different ways for use, not only to better suit the invasive pneumococci in Finland, but also in several other countries or areas, such as Alaska [210], Belgium [159], Brazil [76], Italy [15], and South-Saharan Africa [218]. The flexibility to combine the oligonucleotide primers used for amplification in several ways is important, as it allows the modification of a scheme according to the serotype distribution of the material to be serotyped. The serotype distribution may vary because samples originate from different geographical regions of the world, or from colonising or invasive isolates. PCR-based serotyping is usually less expensive than traditional serotyping, because the reagents used for mPCR are generally available in laboratories and are inexpensive compared to antisera. The most useful and cost-effective mPCR-based serotyping scheme is set up according to the regional serotype distribution, when it is known. When setting up an mPCR-based serotyping scheme, the size of the amplicons should be taken into account, as they must be of sufficiently different sizes to facilitate visual interpretation. Careful handling of samples is essential in mPCR-based serotyping schemes, as well as the use of appropriate negative controls at every stage of the serotyping process. Facilities suitable for PCR work are also crucial and pure cultures must be used for DNA extraction for the results to be reliable. The senisitivity of DNA-based methods is illustrated by a false positive serotype 14 amplicon that was discovered during the validation process. It is likely that this was caused by is a mix up of isolates or contamination at the DNA extraction level. Appropriate negative control practices help to prevent problems associated with contamination. The oligonucleotide primers are not completely serotype specific; some amplify the same size products in all serotypes of a serogroup, such as the primers for serogroup 18. Other primers recognise distinct serotypes that share sequence similarity, such as the primers that amplify serotypes 7B, 7C, and 40 [240]. For accurate serotype deduction, complementary phenotypic methods are needed for the subtyping and confirmation of the serotypes that are not distinguishable due to this kind of primer cross-reactivity, or those serotypes that are not included in any mPCR. Phenotypic serotyping remains the most reliable way to discover possible problems with DNA-based serotyping schemes, as described above in the case of serogroup 19.

One of the benefits of mPCR-based serotype deduction is that isolates can be handled in bulk when isolating DNA and performing mPCRs. This reduces the

required hands-on time per sample. More than 85% of the invasive pneumococci isolated in Finland can be identified either fully or to the serogroup level in the first two mPCRs meaning that the hands-on time per isolate is reasonable. The single disadvantage when handling samples in bulk is the delay in obtaining serotyping results for isolates with rare serotypes identified in mPCRs 5 and 6. The delay is particularly noticeable if only full mPCR runs are performed. Schemes that include detection of *cpsA*, such as the mPCR-based serotyping protocol set up in this study, have the added benefit of confirming the presence of the *cps* genes. This allows for circumvention of downregulated capsule production, which can sometimes be problematic when employing phenotypic methods [278]. Possible untypical *cps* loci will not be detected, however, as the oligonucleotide primers may not be able to attach to the DNA. Because of the indirect nature of the mPCR-based serotyping schemes, it may be beneficial to verify a subset of the results by phenotypic methods, such as Quellung, to make sure the results are in harmony. The reliable interpretation of serotyping reactions by the Quellung reaction requires experience and the regular application of these skills.

6.4 Antimicrobial resistance and macrolide-resistance determinants

The proportion of isolates non-susceptible to penicillin or erythromycin increased significantly over the ten year period. The proportion of penicillin-non-susceptible isolates reached nearly 22% in 2011, i.e. the increase was four-fold compared to the years 1999 to 2000 [251]. The proportion of penicillin-non-susceptible isolates (MIC ≥0.12 mg/L) is higher in Finland than in several other countries. After PCV7 implementation in Italy, non-susceptibility was 14.4% in 2006-2010 [107]. Similarly, the penicillin-non-susceptibility in Finland is very high compared to Sweden, where it was only 3.1% in 2007-2011 [91]. In the whole ten year period, especially the proportion of intermediately penicillin-resistant isolates was higher in Finland than in the other Nordic countries [91]. In Italy in 2006-2010, the proportion of penicillin-resistant isolates (MIC ≥4 mg/L) was 1.3% [107], which is higher than among the Finnish isolates during the same time. However, in stark contrast, the proportion of isolates with penicillin MICs ≥2 mg/L is higher in Finland than in the neighbouring country Sweden, where it was only 1.6% in 2007-2011 [91].

In the first five years of the ten year study period, the proportion of isolates non-susceptibile to erythromycin increased from 16% to 28% among the invasive isolates. Compared to the years 1999 to 2000, the increase was four-fold [251]. Over the last five years of the ten year period, the non-susceptibility to erythromycin remained high but stable at 26.6%, suggesting that the increase observed in the first

five years of the study period levelled off, even though high levels of non-susceptibility were sustained.

Globally, the highest erythromycin-resistance prevalence is found in the Far East, where it was as high as 80% in 2001 to 2004 in a study that included both invasive and non-invasive isolates [101]. While the non-susceptibility of invasive isolates is much lower in Finland, it is again worryingly high compared to the other Nordic countries Denmark, Iceland, Norway, and Sweden. For example, in Sweden, the erythromycin-non-susceptibility was 4.8% in 2007-2011 [91]. The situation in Finland is more comparable to that in Southern Europe [91]. Definitions of high erythromycin MICs vary from ≥32 to 120 mg/L. More than half of the erythromycin-non-susceptible invasive isolates from 2007 to 2011 have an erythromycin MIC ≥64 mg/L; this is likely to be clinically important and may lead to treatment failures [190, 291]. Even if the clinical significance of non-susceptibility is still debated, it seems clear that non-susceptibility allows isolates to proliferate under antimicrobial selection pressure and may give them the opportunity to develop full resistance through recombination. Similar to non-susceptibility levels to penicillin and erythromycin, antimicrobial consumption is higher in Finland than in other Nordic countries, according to a European study [91, 268]. The Nordic countries may have been able to keep their resistance and non-susceptibility levels low at least partly thanks to conservative antimicrobial prescription policies, which help lower the antimicrobial selection pressure in the community [268, 320]. Resistance prevalence and high antimicrobial use may lead to a vicious circle, where each circumstance feeds the increase of the other.

Th proportion of ceftriaxone-non-susceptible pneumococci remained fairly low among the invasive isolates in Finland during the whole study period. The main reason for this is most likely that the proportion of fully penicillin-resistant pneumococci is still relatively low. Ceftriaxone-resistance is more common where highly penicillin-resistant isolates are abundant [94]. In countries with high penicillin-resistance prevalence, such as in Taiwan and Spain, pneumococcal non-susceptibility to ceftriaxone is reported to be 7-9% [50, 310], while in countries with a lower penicillin-resistance prevalence, such as in Italy, Hungary, and Portugal, the respective proportion is around or below 1% [78, 116, 217]. The relationship between penicillin- and ceftriaxone-non-susceptibility is not always a simple one. In Spain, the ceftriaxone-non-susceptibility decreased, although the penicillin-non-susceptibility increased from 1994 to 2004 [78].

The proportion of multidrug-resistant isolates remained stable among the invasive pneumococci over the ten year study period, although both penicillin- and erythromycin-non-susceptibility increased. Multidrug-resistance was defined as non-susceptibility to penicillin, erythromycin, and tetracycline, and the slight decrease in

tetracycline-non-susceptibility seen during 2002-2011 may have tempered the multidrug-resistance proportion. The stability of the multidrug-resistance prevalence is a more hopeful situation than for example in the USA, where the rate of growth of multidrug-resistance in respiratory tract infections in 1992 to 2001 was higher than that of single antibiotic resistance [207]. In the PCV7 era the multidrug-resistance has decreased in the USA [291]. In general, the higher the resistance to erythromycin or penicillin among pneumococci, the higher the prevalence of multidrug-resistance. For example in the USA, multidrug-resistance proportions range from 9% to 25% by region and seems to correspond with the proportion of penicillin- and erythromycin-non-susceptibility [63, 155]. It is worth noting, however, that the specimen types in the aforementioned US studies also included non-invasive pneumococci.

Direct comparisons of antimicrobial resistance percentages between studies are not necessarily straightforward, as the proportions may be affected by the time-period, the use of PCVs, and the breakpoints used, as well as the isolate inclusion criteria. Further, the lack of a uniform definition of pneumococcal multidrug-resistance also makes comparison difficult.

It has been put forth that a subset of non-invasive isolates should be studied for comprehensive surveillance of resistance, as the same pneumococcal isolates are able to cause both invasive and non-invasive disease [175]. The Finnish Study Group for Antimicrobial Resistance (FiRe) monitors the susceptibility of pneumococci regardless of the specimen type and they report increasing non-susceptibility to all examined antimicrobials in 1997-2010 [115]. The non-invasive isolates are not regularly serotyped or genotyped, and Publication II of this study, focusing on a subset of multidrug-resistant non-invasive isolates, is an attempt to broaden the view of the clonality of drug-resistant pneumococci in Finland. The study of multidrug-resistant non-invasive isolates showed that highly resistant serotype 19A isolates were established in the pneumococcal population in Finland already prior to large-scale vaccination. Indeed, three years after the non-invasive isolates had been isolated and roughly one year after PCV10 had been included in the Finnish national vaccination programme, the first highly resistant invasive serotype 19A isolate part of the same genetic lineage was discovered (Publication IV). Isolates and characteristics that first appear among non-invasive isolates are likely to appear also among the invasive isolates before long.

The hypothetical PCV7 serotype coverage exceeded 80% among both the penicillin- and erythromycin-non-susceptible invasive isolates in the years 2002 to 2006, and the PCV10 coverage of non-susceptible isolates was even higher in the years 2007 to 2011. These results indicate that large-scale vaccination that begun in September

2010, with the inclusion of PCV10 in the national vaccination programme, probably will reduce non-susceptibility rates in the coming years. Indeed, some of the more recent antimicrobial susceptibility results presented in this study, as well as by others [171], give reason for cautious hope. Over the whole ten year period in the current study, the non-susceptibility proportions to penicillin and erythromycin were high in the youngest age-groups. However, in 2011, the last year of the study, the proportions of isolates non-susceptibile to erythromycin and penicillin were lower compared to the previous year among isolates from children aged 2 years or younger. Many in this age-group are eligible for vaccination and the slight decline in the proportion of resistant isolates may be a consequence of PCV10 use. Future surveillance will be able to determine if this decrease continues and to which extent it will be reflected in the other age-groups. It is also worth noting that the proportion of non-susceptible isolates may vary slightly from one year to the next, as they have done before. This emphasises that it is too early to draw definite conclusions about the effect of the vaccine on resistance in Finland. Studies from several other countries report declining non-susceptibility proportions following PCV use, especially when it comes to penicillin [60, 70, 291]. However, other reports suggest that the proportion of non-susceptibility to antimicrobial agents has remained stable or increased in the PCV era, sometimes even in the age-group eligible for vaccination [77, 82, 106, 107, 146, 185, 206, 209]. In the long-term, the effect of PCVs on resistance may be a short-lived decrease followed by a rebound [206, 291]. The prevalence of antimicrobial resistance may rapidly increase in non-vaccine serotype pneumococci [98, 149]. The current study shows that non-susceptible non-vaccine serotype isolates are already circulating in the pneumococcal population in Finland. Although their numbers are low, they may increase in the PCV10 era.

The *mef* gene was the most frequently discovered macrolide-resistance determinant among the studied invasive isolates from 2002 to 2006, which is in agreement with the previous studies conducted in Finland [251, 266]. Interestingly, the distribution of macrolide-resistance determinants in Finland is more comparable, or even nearly identical, to that of North America and Scotland, while differing from much of continental Europe [97, 334, 335]. The serotype and susceptibility pattern to antimicrobial agents of many of the Finnish *mef*(A) positive isolates suggest that they may be part of one clone. A previous study has shown that a tenth of the erythromycin-non-susceptible invasive pneumococci in Finland carry a mutation in the ribosome or ribosomal protein that provides macrolide resistance [251]. The presence of ribosomal mutations was not investigated among the isolates in this study, but 13% of macrolide-non-susceptible pneumococci selected for resistance determinant study were not positive for either the known efflux or methylase genes and may therefore carry ribosomal mutations.

6.5 Genotype clonality and pilus-encoding islets

It is clear that the most important CCs among the penicillin-resistant invasive pneumococcal population in Finland are CC156 and CC320, regardless of whether the pre-2008 CLSI breakpoint or the EUCAST breakpoint for penicillin-resistance is used. Members of these two CCs are consistently found among the studied isolates over the whole ten year period and also feature strongly among the non-invasive multidrug-resistant isolates studied.

6.5.1 Clonal complex 156

The largest single CC in the MLST database, CC156, has grown to include several previous independent CCs in recent years [227]. This is illustrated by the fact that CC156 now also covers CC90 and CC138, which were identified among the genotyped isolates of Publication I, and were independent CCs at the time of writing the original manuscript. Whether or not the eBURST algorithm is successful in its goal to group together only STs descended from a common ancestor stain has been questioned, especially when it comes to CC156 [221]. The pneumococcus is unrestrained in donating and incorporating DNA [67, 337], and intraclonal variants have also been shown to have differing invasive disease potential [36]. CC156 contains isolates that are so dissimilar that they may not share any alleles in the seven sequenced housekeeping gene loci [75, 227]. More thorough genotyping methods that include up to 96 different loci are able to distinguish ten different lineages (a-j) within CC156. The discovery of the fairly rare genotype, ST4945, has been pinpointed as the culprit for merging previous CCs, yet analysis clearly indicated that this ST belongs to lineage h within CC156 [221]. The genotypes identified in the Finnish penicillin-resistant material are mainly concentrated within the lineage termed i. This is the lineage which includes the predicted primary CC founder ST156. This CC and its predicted primary founder PMEN3 Spain^{9V}ST156 are important in several other countries as well. Close to half of the penicillin-non-susceptible isolates in Poland in the years 2003 to 2005 and in Sweden in 2003 were related to PMEN3 Spain^{9V}ST156 [273, 284]. A double locus variant of the PMEN clone, ST143, is important both in Finland and in Poland [274]. CC156 is also strongly represented among the intermediately but not fully penicillin-resistant isolates studied. ST671 was the most frequent ST among the penicillin-intermediate isolates. This underlines the different ST distribution compared to the penicillin-resistant isolates, where the most frequent genotype was ST156. In addition, ST671 among the intermediate, ST143 among the fully resistant isolates, and ST2916 found in both groups, as well as several other CC156 isolates, display non-susceptibility to a greater number of antimicrobial agents than the primary founder strain PMEN3 Spain^{9V}ST156.

6.5.2 Clonal complex 320

Among the genetic lineages and serotypes discovered in the non-invasive isolate collection (Publication II), CC320 and serogroup 19 dominates, covering two thirds of all studied isolates. This CC also has a strong presence among the invasive penicillin-resistant isolates (Publications I and IV). The CC320 isolates are related to previously known drug-resistant serogroup 19 isolates, such as the global PMEN14 Taiwan^{19F}ST236 clone [204]. CC320 isolates often are multidrug-resistant and display high penicillin MICs [291], and this may explain their near absence among the intermediate but not fully resistant isolates. The isolates carrying double macrolide-resistance determinants in this study were all part of CC320 (Publications I and II). The members of this genetic lineage also tend to carry both pilus-encoding islets, which may give them a competitive advantage in colonisation, and through that in opportunities to cause disease [18, 230]. CC320 is implicated in the rise of multidrug-resistance following large-scale use of PCV7 in the USA, Spain, and other countries [8, 21, 256]. After some years of PCV7 use in the USA, the serotype 19A prevalence among invasive cases stabilised at a higher level than in the pre-PCV7 era. However, CC320 continued to increase in proportion among the serotype 19A isolates, and along with the success of this clone, the multidrug-resistance within the serotype increased [21]. In contrast, in Norway, where the antimicrobial resistance levels are low, the invasive serotype 19A disease following PCV implementation was found to be caused by the success of a penicillin-susceptible clone [320]. In some countries, including Finland, as shown in this study, as well as Israel, Korea, and Taiwan, members of the CC320 serotype 19A clone were present already before or during low PCV use [69, 144, 281]. This finding implies that selection pressure from antibiotics is an important factor in the success of the clone. It is likely that unless PCV10 provides cross-protection against serotype 19A, this clone will be sustained in the PCV10 era in Finland and may impact the multidrug-resistance situation. Some members of CC320 or serotype 19A display capsular switching within serogroup 19 [227] and even outside the serogroup [4, 338]. The serotype 19A ST320 clone that has been widely encountered globally appears to have evolved from its ancestral clone PMEN14 Taiwan^{19F}ST236 through serotype switching. Other changes have also taken place in the genome that allow ST320 to outcompete the parent strain in colonisation experiments [144]. Compared to serotype 19F ST320, the serotype 19A variant carries similar *pbp*, *erm*(B), *mef*(A/E), and *tetM* genes, but has additional mutations in several other genes that are connected with antimicrobial resistance or virulence [256].

6.5.3 Novel sequence types and other clonal complexes

As many as four novel *ddl* genes were found among the subset of invasive isolates studied by genotyping. The *ddl* gene is situated only 783 bp downstream from the *pbp2b* gene and known to be especially prone to variations during the development

of penicillin-resistance. Interspecies recombination at the *pbp2b* locus often occurs under selection pressure brought on by the use of penicillin, and the changes in the genome often extend through the *ddl* gene. This so-called gene hitchhiking has been described extensively and is the reason for eliminating the locus from some tools for the analysis of pneumococcal clonality, as it may distort the relationship between strains [89].

Of the penicillin-resistant isolates genotyped in 2002 to 2006, 16% had STs that have to date not been described elsewhere, and at the time of writing the manuscript this proportion covered as many as a third of the isolates. An even higher proportion was described in a study from Norway, where 42% of the genotyped penicillin-non-susceptible isolates had a previously unknown genotype [290]. Interestingly, with one exception (ST2918), the novel genotypes described among these Finnish isolates are multidrug-resistant and many of them are also related to successful international clones. The time and place of origin of the novel STs detected in this study is unknown. The MLST database relies on the voluntary submission of genotyped isolates and is thus unlikely to include all analysed isolates. Furthermore, it is probable that a bias towards studying drug-resistant and invasive pneumococci is reflected as oversampling in the database material. Genotyping may also not be performed by the same frequency everywhere. These considerations combined with the limitations of the eBURST algorithm may also affect the assignment of both subgroup founders and the predicted primary founder of a CC [75]. For instance, it is likely that the true founder of CC156, and of lineage i within it, is the penicillin-susceptible genotype ST162. However, the algorithm assigns ST156 as the predicted primary founder of the CC [293]. ST162 has nearly as many SLVs as ST156, and more double and triple locus variants, which places this genotype at the centre of CC156 [75].

While several of the same CCs were present among the intermediately penicillin-resistant isolates from the year 2005 as among the penicillin-resistant isolates, there are marked differences in the distribution of STs within the populations. It is interesting to note that several of the PMEN strains, such as PMEN31 Netherlands^{3}ST180 or PMEN9 England^{14}ST9, identified or related to isolates among the intermediately resistant isolates are susceptible to penicillin. Serotypes, such as 3, 6A, 9N, and 18C, which rarely exhibit penicillin-non-susceptibility, are represented by single or a couple of isolates. These observations indicate recombination of non-susceptible isolates, in addition to the presence of clonal non-susceptibility. These kinds of isolates may become significant especially in the PCV10 era as several of them are not covered by the vaccine. Antimicrobial resistance determinants such as the altered *pbp* genes required for penicillin-resistance have been introduced through horizontal gene transfer after the CCs have been established. This is illustrated by the fact that the CCs also contain susceptible strains [337]. Taken together, this

indicates that established and successful clones may acquire resistance determinants and survive in a setting with antimicrobial selection pressure.

Although the serotype is not a completely reliable clonal marker, the increasing levels of penicillin- and erythromycin-non-susceptibility among serotype 14 isolates in this study, suggests that clonal expansion may be a driving factor in intermediate resistance. Intermediately penicillin-resistant and erythromycin-non-susceptible serotype 14 isolates were represented in the material by CC156, CC63, and CC15. These are important widely disseminated CCs and CC15 has a high invasive disease potential [36, 227], therefore it is likely that they are present among the invasive pneumococci in Finland also in the later years.

6.6 Pilus-encoding islets

Two pneumococcal pili encoded by PI-1 and PI-2 have been described on the surface of pneumococci and have been suggested as potential vaccine candidates for a multivalent protein vaccine against pneumococcal disease [16, 223]. In a mouse model, this pilus provided a competitive advantage over non-piliated pneumococci in the nasopharynx, and it has been suggested that this may contribute to the spread of the piliated clones [284]. The presence of PI-1 has previously been shown to correlate with antimicrobial resistance [2], but no consistent similar association has been confirmed for PI-2. However, in a study conducted in Atlanta, Georgia, USA, in the PCV7 era, all the PI-2 positive serogroup 19 isolates in CC320 were resistant to penicillin, erythromycin, trimethoprim/sulfamethoxazole, tetracycline, and chloramphenicol [343]. This resembles the non-susceptibility pattern of the CC320 isolates from Southern Finland and of the majority of the invasive CC320 isolates for the antimicrobial agents tested. Members of CC320 generally carry both PI-1 and PI-2.

Among the invasive pneumococci, the PI-1 positive genotypes were found in five CCs, including CC156 and CC320, discussed above, and in one singleton. Carriage of PI-1 has previously been found to associate both with the serotype and the genotype, and PI-1 gene prevalence is high among drug-resistant isolates [2, 19, 223]. In isolates from Portugal, PMEN3 Spain^{9V}ST156 is associated with the presence of PI-1, regardless of serotype and 61% of the penicillin-non-susceptible isolates carried PI-1, while the corresponding figure for susceptible isolates was 16% [2].

In a Finnish study into non-invasive isolates from the early 1990s, 20% of the pneumococci isolated from children with acute otitis media carried the PI-1 genes, but the PI-2 genes were not found [308]. In contrast, the large proportion of PI-2

positive non-invasive and invasive isolates in this study, which focuses on clonality with reference to antimicrobial resistance, may suggest that PI-2 could have spread the pneumococcal population in tandem with resistant clones.

The frequency of PI-1 has reportedly initially decreased since the introduction of PCV7, probably because a majority of the PI-1 associated serotypes described so far are covered by the available vaccines [19]. Recently it has been reported that PI-1 re-emerged after a few years of PCV7 use and in some areas it has eventually exceeded the pre-vaccination rate [267]. The prevalence of PI-1 reported to be approximately 15% to 30% depending on the area and the vaccination programme status in the community [1, 222, 267, 279]. On the other hand, reports indicate that PI-2 carrying invasive pneumococci have increased following wide use of PCV7, partly because of the emergence of piliated serotype 19A isolates [1, 343]. In a large study of paediatric isolates from Alabama, USA, the proportion of PI-1, *pspA*, and *pspC* gene carrying isolates was unaffected by seven years of PCV7 use [64]. Overall, 92% of the non-invasive isolates, and 74% (2002-2006) or 100% (2007-2011) of the invasive isolates genotyped in this study carried the genes for one or both pili. Clearly, pilus gene carriage among drug resistant pneumococci in Finland is high, which indicates that a potential future vaccine containing pilus proteins would be helpful in controlling the spread of these clones.

7 Conclusions and future considerations

This study illustrates the importance of surveillance of both clonality and antimicrobial resistance. Large-scale interventions, such as the use of PCV10, which since September 2010 is part of the Finnish national vaccination programme, underlines the importance of ongoing surveillance. The serotyping scheme set up and validated in this study will aid in the continued serotype-specific surveillance of the invasive pneumococcal population. Studying the pneumococcal population by genotyping provides more information about the circulating strains than serotyping alone can provide, and will help to determine if the established clones proliferate or are replaced by others.

This study shows that several globally disseminated resistant clones, such as PMEN3 Spain^{9V}ST156, have established themselves in Finland. These clones also include the multidrug-resistant serotype 19A clone within CC320 that globally has emerged primarily following extensive PCV use. The impact of these clones on the antimicrobial resistance following large-scale vaccination in Finland is still unclear. However, it is likely, that as they are already well-established, these successful resistant clones will be sustained and possibly expand. As seen in this study, clones found among non-invasive isolates, are likely to turn up also in the invasive population before long. Comprehensive surveillance of clonality in relation to antimicrobial resistance should therefore also in the future aim to examine a subset of non-invasive isolates. The novel genotypes in this material attest to the continuous recombination and diversification of existing resistant clones.

The antimicrobial non-susceptibility and resistance levels in Finland are higher than in its neighbouring countries, and increased over the ten year study period 2002-2011. This is worrying, especially as high non-susceptibility levels may lead to a vicious circle, where more antimicrobials are used to treat infections, which in turn often leads to higher resistance. In Finland, the proportion of isolates non-susceptible to penicillin or erythromycin is especially high, covering a fifth and a fourth of the invasive isolates from the year 2011, respectively. PCV10 covers the majority of the non-susceptible isolates among the invasive isolates, which gives hope that the resistance prevalence will be restrained by vaccine use in the coming years. However, some non-vaccine serotypes are also present among the non-

susceptible isolates and these may quickly develop full resistance under selection pressure from antimicrobials.

New typing methods and the increasing use of PCVs are likely to bring about the discovery of novel serotypes. This study shows that some newly identified serotypes, such as 6C and 6D, have been present in the Finnish invasive pneumococcal population for several years. This has implications for potential serotype replacement following PCV use, as these and previously rare serotypes may proliferate when previously successful clones are controlled by vaccines.

To conclude, this study deepens our understanding of the clonality of pneumococci in relation to antimicrobial resistance in Finland and illustrates the dynamic nature of the pneumococcal population. Due to the years included in this study, the main focus is on the pneumococci isolated before extensive PCV use, but future monitoring will be able to determine how the population evolves in the PCV era. It is essential that the surveillance of both serotype clonality and antimicrobial resistance continues in the PCV era.

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9 References

1. Aguiar SI, Melo-Cristino J, Ramirez M. Use of the 13-valent conjugate vaccine has the potential to eliminate pilus carrying isolates as causes of invasive pneumococcal disease. Vaccine 2012;30(37):5487-5490.
2. Aguiar SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M. The presence of the pilus locus is a clonal property among pneumococcal invasive isolates. BMC Microbiol 2008;8(1):41.
3. AlonsoDeVelasco E, Verheul AF, Verhoef J, Snippe H. *Streptococcus pneumoniae*: virulence factors, pathogenesis, and vaccines. Microbiol Rev 1995;59(4):591-603.
4. Ansaldi F, Canepa P, de Florentiis D, Bandettini R, Durando P, Icardi G. Increasing incidence of *Streptococcus pneumoniae* serotype 19A and emergence of two vaccine escape recombinant ST695 strains in Liguria, Italy, 7 years after implementation of the 7-valent conjugated vaccine. Clin Vaccine Immunol 2011;18(2):343-5.
5. Appelbaum PC. Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. Clin Infect Dis 1992;15(1):77-83.
6. Arason VA, Sigurdsson JA, Erlendsdottir H, Gudmundsson S, Kristinsson KG. The role of antimicrobial use in the epidemiology of resistant pneumococci: A 10-year follow up. Microb Drug Resist 2006;12(3):169-76.
7. Arason VA, Gunnlaugsson A, Sigurdsson JA, Erlendsdottir H, Gudmundsson S, Kristinsson KG. Clonal spread of resistant pneumococci despite diminished antimicrobial use. Microb Drug Resist 2002;8(3):187-192.
8. Ardanuy C, Rolo D, Fenoll A, Tarrago D, Calatayud L, Linares J. Emergence of a multidrug-resistant clone (ST320) among invasive serotype 19A pneumococci in Spain. J. Antimicrob. Chemother. 2009;64(3):507-510.
9. Aristegui J, Bernaola E, Pocheville I, Garcia C, Arranz L, Duran G, Perez L, Bastida M, Canduela C, Herranz Aguirre M, Garrote E, Fletcher MA, Perez C. Reduction in pediatric invasive pneumococcal disease in the Basque Country and Navarre, Spain, after introduction of the heptavalent pneumococcal conjugate vaccine. Eur J Clin Microbiol Infect Dis 2007;26(5):303-310.
10. Arrecubieta C, Garcia E, Lopez R. Sequence and transcriptional analysis of a DNA region involved in the production of capsular polysaccharide in *Streptococcus pneumoniae* type 3. Gene 1995;167(1-2):1-7.
11. Austrian R. Pneumococcus: the first one hundred years. Rev Infect Dis 1981;3(2):183-189.
12. Austrian R. The quellung reaction, a neglected microbiologic technique. Mt Sinai J Med 1976;43(6):699-709.
13. Austrian R, Bernheimer HP, Smith EE, Mills GT. Simultaneous production of two capsular polysaccharides by pneumococcus. II. The genetic and biochemical bases of binary capsulation. J Exp Med 1959;110:585-602.

14. Avery OT, MacLeod CM, McCarty M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J Exp Med 1944;79(2):137-158.
15. Azzari C, Moriondo M, Indolfi G, Massai C, Becciolini L, de Martino M, Resti M. Molecular detection methods and serotyping performed directly on clinical samples improve diagnostic sensitivity and reveal increased incidence of invasive disease by *Streptococcus pneumoniae* in Italian children. J Med Microbiol 2008;57(Pt 10):1205-1212.
16. Bagnoli F, Moschioni M, Donati C, Dimitrovska V, Ferlenghi I, Facciotti C, Muzzi A, Giusti F, Emolo C, Sinisi A, Hilleringmann M, Pansegrau W, Censini S, Rappuoli R, Covacci A, Masignani V, Barocchi MA. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. J Bacteriol 2008;190(15):5480-92.
17. Bardossi F. Oswald Avery and the sugar-coated microbe. The Rockefeller University Research Profiles 1988;Spring 1988(31).
18. Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, Dahlberg S, Fernebro J, Moschioni M, Masignani V, Hultenby K, Taddei AR, Beiter K, Wartha F, von Euler A, Covacci A, Holden DW, Normark S, Rappuoli R, Henriques-Normark B. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci U S A 2006;103:2857-2862.
19. Basset A, Trzcinski K, Hermos C, O'Brien KL, Reid R, Santosham M, McAdam AJ, Lipsitch M, Malley R. Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence. J Clin Microbiol 2007;45(6):1684-9.
20. Batt SL, Charalambous BM, McHugh TD, Martin S, Gillespie SH. Novel PCR-restriction fragment length polymorphism method for determining serotypes or serogroups of *Streptococcus pneumoniae* isolates. J Clin Microbiol 2005;43(6):2656-61.
21. Beall BW, Gertz RE, Hulkower RL, Whitney CG, Moore MR, Brueggemann AB. Shifting genetic structure of invasive serotype 19A pneumococci in the United States. J Infect Dis 2011;203(10):1360-1368.
22. Benfield T, Skovgaard M, Schonheyder HC, Knudsen JD, Bangsborg J, Ostergaard C, Slotved HC, Konradsen HB, Thomsen RW, Lambertsen L. Serotype distribution in non-bacteremic pneumococcal pneumonia: association with disease severity and implications for pneumococcal conjugate vaccines. PLoS One 2013;8(8):e72743.
23. Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, Collins M, Donohoe K, Harris D, Murphy L, Quail MA, Samuel G, Skovsted IC, Kaltoft MS, Barrell B, Reeves PR, Parkhill J, Spratt BG. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet 2006;2(3):e31.
24. Berg KH, Biornstad TJ, Johnsborg O, Håvarstein LS. Properties and biological role of streptococcal fratricins. Appl Environ Microbiol 2012;78(10):3515-3522.

25. Bergman M, Huikko S, Huovinen P, Paakkari P, Seppälä H. Macrolide and azithromycin use are linked to increased macrolide resistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 2006;50(11):3646-50.
26. Bernheimer HP, Wermundsen IE. Unstable binary capsulated transformants in pneumococcus. J Bacteriol 1969;98(3):1073-1079.
27. Birtles A, McCarthy N, Sheppard CL, Rutter H, Guiver M, Haworth E, George RC. Multilocus sequence typing directly on DNA from clinical samples and a cultured isolate to investigate linked fatal pneumococcal disease in residents of a shelter for homeless men. J Clin Microbiol 2005;43(4):2004-8.
28. Bishop CJ. Pneumococcal MLST oligonucleotide primer sequence, personal communication by email 30th January, 2007.
29. Blasi F, Mantero M, Santus P, Tarsia P. Understanding the burden of pneumococcal disease in adults. Clin Microbiol Infect 2012;18 Suppl 5:7-14.
30. Bogaert D, Keijser B, Huse S, Rossen J, Veenhoven R, van Gils E, Bruin J, Montijn R, Bonten M, Sanders E. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. PLoS One 2011;6(2):e17035.
31. Bogaert D, De Groot R, Hermans PW. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis 2004;4(3):144-154.
32. Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rumke HC, Verbrugh HA, Hermans PW. Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. Lancet 2004;363(9424):1871-1872.
33. Bowers EF, Jeffries LR. Optochin in the identification of *Str. pneumoniae*. J. clin. Path. 1955;8:58-60.
34. Bratcher PE, Kim KH, Kang JH, Hong JY, Nahm MH. Identification of natural pneumococcal isolates expressing serotype 6D by genetic, biochemical and serological characterization. Microbiology 2010;156(Pt 2):555-60.
35. Bratcher PE, Park IH, Hollingshead SK, Nahm MH. Production of a unique pneumococcal capsule serotype belonging to serogroup 6. Microbiology 2009;155(Pt 2):576-583.
36. Browall S, Norman M, Tangrot J, Galanis I, Sjöström K, Dagerhamn J, Hellberg C, Pathak A, Spadafina T, Sandgren A, Battig P, Franzen O, Andersson B, Örtqvist A, Normark S, Henriques-Normark B. Intraclonal variations among *Streptococcus pneumoniae* isolates influence the likelihood of invasive disease in children. J Infect Dis 2013;[Epub ahead of print] 10.1093/infdis/jit481.
37. Brueggemann AB, Pai R, Crook DW, Beall B. Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. PLoS Pathog 2007;3(11):e168.
38. Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, Spratt BG. Temporal and geographic stability of the serogroup-specific invasive disease potential of *Streptococcus pneumoniae* in children. J Infect Dis 2004;190(7):1203-1211.
39. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. J Infect Dis 2003;187(9):1424-1432.

40. Burgos J, Falco V, Pahissa A. The increasing incidence of empyema. Curr Opin Pulm Med 2013;19(4):350-356.
41. Byington CL, Korgenski K, Daly J, Ampofo K, Pavia A, Mason EO. Impact of the pneumococcal conjugate vaccine on pneumococcal parapneumonic empyema. Pediatr Infect Dis J 2006;25(3):250-254.
42. Calix JJ, Porambo RJ, Brady AM, Larson TR, Yother J, Abeygunwardana C, Nahm MH. Biochemical, genetic, and serological characterization of two capsule subtypes among *Streptococcus pneumoniae* serotype 20 strains: discovery of a new pneumococcal serotype. J Biol Chem 2012;287(33):27885-27894.
43. Calix J, Nahm M. A new pneumococcal serotype, 11E, has a variably inactivated *wcjE* gene. J Infect Dis 2010;202(1):29-38.
44. Carroll L. Alice's Adventures in Wonderland and Through the Looking Glass and What Alice Found There. London, UK: Penguin Classics; 2009.
45. Carvalho Mda G, Bigogo GM, Junghae M, Pimenta FC, Moura I, Roundtree A, Li Z, Conklin L, Feikin DR, Breiman RF, Whitney CG, Beall B. Potential nonpneumococcal confounding of PCR-based determination of serotype in carriage. J Clin Microbiol 2012;50(9):3146-3147.
46. Carvalho Mda G, Pimenta FC, E. GR,Jr, Joshi HH, Trujillo AA, Keys LE, Findley J, Moura IS, Park IH, Hollingshead SK, Pilishvili T, Whitney CG, Nahm MH, Beall BW. PCR-based quantitation and clonal diversity of the current prevalent invasive serogroup 6 pneumococcal serotype, 6C, in the United States in 1999 and 2006 to 2007. J Clin Microbiol 2009;47(3):554-9.
47. Centers for Disease Control and Prevention (CDC). Progress in introduction of pneumococcal conjugate vaccine - worldwide, 2000-2012. MMWR Morb Mortal Wkly Rep 2013;62(16):308-311.
48. Centers for Disease Control and Prevention (CDC). Effects of new penicillin susceptibility breakpoints for *Streptococcus pneumoniae*--United States, 2006-2007. MMWR Morb Mortal Wkly Rep 2008;57(50):1353-1355.
49. Chien YW, Klugman KP, Morens DM. Efficacy of whole-cell killed bacterial vaccines in preventing pneumonia and death during the 1918 influenza pandemic. J Infect Dis 2010;202(11):1639-1648.
50. Chiu CH, Su LH, Huang YC, Lai JC, Chen HL, Wu TL, Lin TY. Increasing ceftriaxone resistance and multiple alterations of penicillin-binding proteins among penicillin-resistant *Streptococcus pneumoniae* isolates in Taiwan. Antimicrob Agents Chemother 2007;51(9):3404-6.
51. Choi EH, Lee HJ, Cho EY, Oh CE, Eun BW, Lee J, Kim MJ. Prevalence and genetic structures of *Streptococcus pneumoniae* serotype 6D, South Korea. Emerg Infect Dis 2010;16(11):1751-1753.
52. Ciruela P, Soldevila N, Hernandez S, Selva L, de Sevilla MF, Garcia-Garcia JJ, Moraga F, Planes AM, Munoz-Almagro C, Dominguez A. Risk factors for invasive pneumococcal disease in a community with a high proportion of non vaccine serotypes. Vaccine 2013;31(6):960-966.
53. Ciruela P, Soldevila N, Selva L, Hernandez S, Garcia-Garcia JJ, Moraga F, de Sevilla MF, Codina G, Planes AM, Esteva C, Coll F, Cardenosa N, Jordan I, Batalla J, Salleras L, Munoz-Almagro C, Dominguez A. Are risk factors

associated with invasive pneumococcal disease according to different serotypes? Hum Vaccin Immunother 2013;9(3).
54. Claverys JP, Martin B, Håvarstein LS. Competence-induced fratricide in streptococci. Mol Microbiol 2007;64(6):1423-1433.
55. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: 18th informational supplement. CLSI document M100-S18. Wayne, PA, USA: Clinical and Laboratory Standards Institute; 2008.
56. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: 15th informational supplement. Pennsylvania, USA: Clinical and Laboratory Standards Institute; 2005.
57. Coffey TJ, Daniels M, Enright MC, Spratt BG. Serotype 14 variants of the Spanish penicillin-resistant serotype 9V clone of *Streptococcus pneumoniae* arose by large recombinational replacements of the *cpsA-pbp1a* region. Microbiology 1999;145 (Pt 8)(Pt 8):2023-2031.
58. Cohen R, Levy C, Bingen E, Koskas M, Nave I, Varon E. Impact of 13-valent pneumococcal conjugate vaccine on pneumococcal nasopharyngeal carriage in children with acute otitis media. Pediatr Infect Dis J 2012;31(3):297-301.
59. Cooper D, Yu X, Sidhu M, Nahm MH, Fernsten P, Jansen KU. The 13-valent pneumococcal conjugate vaccine (PCV13) elicits cross-functional opsonophagocytic killing responses in humans to *Streptococcus pneumoniae* serotypes 6C and 7A. Vaccine 2011;29(41):7207-7211.
60. Cornick JE, Bentley SD. *Streptococcus pneumoniae*: the evolution of antimicrobial resistance to beta-lactams, fluoroquinolones and macrolides. Microbes Infect 2012;14(7-8):573-583.
61. Corso A, Severina EP, Petruk VF, Mauriz YR, Tomasz A. Molecular characterization of penicillin-resistant *Streptococcus pneumoniae* isolates causing respiratory disease in the United States. Microb Drug Resist 1998;4(4):325-337.
62. Crisafulli G, Guidotti S, Muzzi A, Torricelli G, Moschioni M, Masignani V, Censini S, Donati C. An extended multi-locus molecular typing schema for *Streptococcus pneumoniae* demonstrates that a limited number of capsular switch events is responsible for serotype heterogeneity of closely related strains from different countries. Infect Genet Evol 2013;13:151-161.
63. Critchley IA, Brown SD, Traczewski MM, Tillotson GS, Janjic N. National and regional assessment of antimicrobial resistance among community-acquired respiratory tract pathogens identified in a 2005-2006 U.S. Faropenem surveillance study. Antimicrob Agents Chemother 2007;51(12):4382-9.
64. Croney CM, Nahm MH, Juhn SK, Briles DE, Crain MJ. Invasive and noninvasive *Streptococcus pneumoniae* capsule and surface protein diversity following use of conjugate vaccine. Clin Vaccine Immunol 2013.
65. Croucher NJ, Finkelstein JA, Pelton SI, Mitchell PK, Lee GM, Parkhill J, Bentley SD, Hanage WP, Lipsitch M. Population genomics of post-vaccine changes in pneumococcal epidemiology. Nat Genet 2013.

66. Croucher NJ, Harris SR, Barquist L, Parkhill J, Bentley SD. A high-resolution view of genome-wide pneumococcal transformation. PLoS Pathog 2012;8(6):e1002745.
67. Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, McGee L, von Gottberg A, Song JH, Ko KS, Pichon B, Baker S, Parry CM, Lambertsen LM, Shahinas D, Pillai DR, Mitchell TJ, Dougan G, Tomasz A, Klugman KP, Parkhill J, Hanage WP, Bentley SD. Rapid pneumococcal evolution in response to clinical interventions. Science 2011;331(6016):430-434.
68. da Gloria Carvalho M, Pimenta FC, Jackson D, Roundtree A, Ahmad Y, Millar EV, O'Brien KL, Whitney CG, Cohen AL, Beall BW. Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J Clin Microbiol 2010;48(5):1611-1618.
69. Dagan R, Givon-Lavi N, Leibovitz E, Greenberg D, Porat N. Introduction and proliferation of multidrug-resistant *Streptococcus pneumoniae* serotype 19A clones that cause acute otitis media in an unvaccinated population. J Infect Dis 2009;199(6):776-85.
70. Dagan R, Klugman KP. Impact of conjugate pneumococcal vaccines on antibiotic resistance. The Lancet Infectious Diseases 2008;8(12):785-795.
71. Dagerhamn J, Blomberg C, Browall S, Sjöström K, Morfeldt E, Henriques-Normark B. Determination of accessory gene patterns predicts the same relatedness among strains of *Streptococcus pneumoniae* as sequencing of housekeeping genes does and represents a novel approach in molecular epidemiology. J Clin Microbiol 2008;46(3):863-868.
72. Daly MM, Doktor S, Flamm R, Shortridge D. Characterization and prevalence of MefA, MefE, and the associated *msr*(D) gene in *Streptococcus pneumoniae* clinical isolates. J Clin Microbiol 2004;42(8):3570-4.
73. Danne C, Dramsi S. Pili of gram-positive bacteria: roles in host colonization. Res Microbiol 2012;163(9-10):645-658.
74. De Wals P, Lefebvre B, Defay F, Deceuninck G, Boulianne N. Invasive pneumococcal diseases in birth cohorts vaccinated with PCV-7 and/or PHiD-CV in the province of Quebec, Canada. Vaccine 2012;30(45):6416-6420.
75. eBURSTv3. Department of Infectious Disease Epidemiology at Imperial College London. Available at: http://eburst.mlst.net/. Accessed 15th September, 2013.
76. Dias CA, Teixeira LM, Carvalho Mda G, Beall B. Sequential multiplex PCR for determining capsular serotypes of pneumococci recovered from Brazilian children. J Med Microbiol 2007;56(Pt 9):1185-8.
77. Dias R, Canica M. Trends in resistance to penicillin and erythromycin of invasive pneumococci in Portugal. Epidemiol Infect 2008;136(7):928-39.
78. Dias R, Louro D, Canica M. Antimicrobial susceptibility of invasive *Streptococcus pneumoniae* isolates in Portugal over an 11-year period. Antimicrob Agents Chemother 2006;50(6):2098-105.
79. Domenech M, Ramos-Sevillano E, Garcia E, Moscoso M, Yuste J. Biofilm formation avoids complement immunity and phagocytosis of *Streptococcus pneumoniae*. Infect Immun 2013.

80. Donati C, Hiller NL, Tettelin H, Muzzi A, Croucher NJ, Angiuoli SV, Oggioni M, Dunning Hotopp JC, Hu FZ, Riley DR, Covacci A, Mitchell TJ, Bentley SD, Kilian M, Ehrlich GD, Rappuoli R, Moxon ER, Masignani V. Structure and dynamics of the pan-genome of *Streptococcus pneumoniae* and closely related species. Genome Biol 2010;11(10):R107-2010-11-10-r107. Epub 2010 Oct 29.
81. Dopazo J, Mendoza A, Herrero J, Caldara F, Humbert Y, Friedli L, Guerrier M, Grand-Schenk E, Gandin C, de Francesco M, Polissi A, Buell G, Feger G, Garcia E, Peitsch M, Garcia-Bustos JF. Annotated draft genomic sequence from a *Streptococcus pneumoniae* type 19F clinical isolate. Microb Drug Resist 2001;7(2):99-125.
82. Dos Santos SR, Passadore LF, Takagi EH, Fujii CM, Yoshioka CR, Gilio AE, Martinez MB. Serotype distribution of *Streptococcus pneumoniae* isolated from patients with invasive pneumococcal disease in Brazil before and after ten-pneumococcal conjugate vaccine implementation. Vaccine 2013.
83. du Plessis M, von Gotteberg A, Madhi SA, Hattingh O, de Gouveia L, Klugman KP. Serotype 6C is associated with penicillin-susceptible meningeal infections in human immunodeficiency virus (HIV)-infected adults among invasive pneumococcal isolates previously identified as serotype 6A in South Africa. Int J Antimicrob Agents 2008;32(Suppl 1):S66-S70.
84. Edelstein PH. Pneumococcal resistance to macrolides, lincosamides, ketolides, and streptogramin B agents: molecular mechanisms and resistance phenotypes. Clin Infect Dis 2004;38 Suppl 4:S322-7.
85. Elberse K, Witteveen S, van der Heide H, van de Pol I, Schot C, van der Ende A, Berbers G, Schouls L. Sequence diversity within the capsular genes of *Streptococcus pneumoniae* serogroup 6 and 19. PLoS One 2011;6(9):e25018.
86. Elberse KE, Nunes S, Sa-Leao R, van der Heide HG, Schouls LM. Multiple-locus variable number tandem repeat analysis for *Streptococcus pneumoniae*: comparison with PFGE and MLST. PLoS One 2011;6(5):e19668.
87. Elberse KE, van de Pol I, Witteveen S, van der Heide HG, Schot CS, van Dijk A, van der Ende A, Schouls LM. Population structure of invasive *Streptococcus pneumoniae* in The Netherlands in the pre-vaccination era assessed by MLVA and capsular sequence typing. PLoS One 2011;6(5):e20390.
88. Enright MC, Knox K, Griffiths D, Crook DW, Spratt BG. Molecular typing of bacteria directly from cerebrospinal fluid. Eur J Clin Microbiol Infect Dis 2000;19(8):627-30.
89. Enright MC, Spratt BG. Extensive variation in the *ddl* gene of penicillin-resistant *Streptococcus pneumoniae* results from a hitchhiking effect driven by the penicillin-binding protein 2b gene. Mol Biol Evol 1999;16(12):1687-95.
90. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology 1998;144 (Pt 11):3049-60.
91. European Antimicrobial Resistance Surveillance Network (EARS-Net), European Centre for Disease Prevention and Control. Available at:

http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net. Accessed 6th September, 2013.
92. European Centre for Disease Prevention and Control. Annual Epidemiological Report 2012. Surveillance reports 2013.
93. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 3.0. 2013;www.eucast.org.
94. Faella F, Pagliano P, Fusco U, Attanasio V, Conte M. Combined treatment with ceftriaxone and linezolid of pneumococcal meningitis: a case series including penicillin-resistant strains. Clin Microbiol Infect 2006;12(4):391-394.
95. Fani F, Brotherton MC, Leprohon P, Ouellette M. Genomic analysis and reconstruction of cefotaxime resistance in *Streptococcus pneumoniae*. J Antimicrob Chemother 2013.
96. Fani F, Leprohon P, Legare D, Ouellette M. Whole genome sequencing of penicillin-resistant *Streptococcus pneumoniae* reveals mutations in penicillin-binding proteins and in a putative iron permease. Genome Biol 2011;12(11):R115-2011-12-11-r115.
97. Farrell DJ, Couturier C, Hryniewicz W. Distribution and antibacterial susceptibility of macrolide resistance genotypes in *Streptococcus pneumoniae*: PROTEKT Year 5 (2003-2004). Int J Antimicrob Agents 2008;31(3):245-9.
98. Farrell DJ, Klugman KP, Pichichero M. Increased antimicrobial resistance among nonvaccine serotypes of *Streptococcus pneumoniae* in the pediatric population after the introduction of 7-valent pneumococcal vaccine in the United States. Pediatr Infect Dis J 2007;26(2):123-8.
99. Feikin DR, Klugman KP. Historical changes in pneumococcal serogroup distribution: implications for the era of pneumococcal conjugate vaccines. Clin Infect Dis 2002;35(5):547-555.
100. Feil EJ, Smith JM, Enright MC, Spratt BG. Estimating recombinational parameters in *Streptococcus pneumoniae* from multilocus sequence typing data. Genetics 2000;154(4):1439-1450.
101. Felmingham D, Canton R, Jenkins SG. Regional trends in beta-lactam, macrolide, fluoroquinolone and telithromycin resistance among *Streptococcus pneumoniae* isolates 2001-2004. J Infect 2007;55(2):111-8.
102. Fenoll A, Jado I, Vicioso D, Casal J. Dot blot assay for the serotyping of pneumococci. J Clin Microbiol 1997;35(3):764-766.
103. Figueira-Coelho J, Ramirez M, Salgado MJ, Melo-Cristino J. *Streptococcus agalactiae* in a Large Portuguese Teaching Hospital: Antimicrobial Susceptibility, Serotype Distribution, and Clonal Analysis of Macrolide-Resistant Isolates. Microb Drug Resist 2004;10(1):31-36.
104. Fleming-Dutra K, Mbaeyi C, Link-Gelles R, Alexander N, Guh A, Forbes E, Beall B, Winchell JM, Carvalho Mda G, Pimenta F, Kodani M, Vanner C, Stevens H, Brady D, Caulcrick-Grimes M, Bandy U, Moore MR. *Streptococcus pneumoniae* serotype 15A in psychiatric unit, Rhode Island, USA, 2010-2011. Emerg Infect Dis 2012;18(11):1889-1893.
105. Fleming-Dutra KE, Taylor T, Link-Gelles R, Garg S, Jhung MA, Finelli L, Jain S, Shay D, Chaves SS, Baumbach J, Hancock EB, Beall B, Bennett N, Zansky

S, Petit S, Yousey-Hindes K, Farley MM, Gershman K, Harrison LH, Ryan P, Lexau C, Lynfield R, Reingold A, Schaffner W, Thomas A, Moore MR. Effect of the 2009 influenza A(H1N1) pandemic on invasive pneumococcal pneumonia. J Infect Dis 2013;207(7):1135-1143.

106. Frazao N, Brito-Avo A, Simas C, Saldanha J, Mato R, Nunes S, Sousa NG, Carrico JA, Almeida JS, Santos-Sanches I, de Lencastre H. Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers in Lisbon. Pediatr Infect Dis J 2005;24(3):243-52.
107. Gherardi G, D'Ambrosio F, Visaggio D, Dicuonzo G, Del Grosso M, Pantosti A. Serotype and clonal evolution of penicillin-nonsusceptible invasive *Streptococcus pneumoniae* in the 7-valent pneumococcal conjugate vaccine era in Italy. Antimicrob Agents Chemother 2012;56(9):4965-4968.
108. Gianfaldoni C, Censini S, Hilleringmann M, Moschioni M, Facciotti C, Pansegrau W, Masignani V, Covacci A, Rappuoli R, Barocchi MA, Ruggiero P. *Streptococcus pneumoniae* pilus subunits protect mice against lethal challenge. Infect Immun 2007;75(2):1059-1062.
109. Granat SM, Mia Z, Ollgren J, Herva E, Das M, Piirainen L, Auranen K, Mäkelä PH. Longitudinal study on pneumococcal carriage during the first year of life in Bangladesh. Pediatr Infect Dis J 2007;26(4):319-324.
110. Grau I, Ardanuy C, Calatayud L, Rolo D, Domenech A, Linares J, Pallares R. Invasive pneumococcal disease in healthy adults: increase of empyema associated with the clonal-type Sweden(1)-ST306. PLoS One 2012;7(8):e42595.
111. Gray BM, Musher DM. The history of pneumococcal disease. In: Siber GR, Klugman KP, Mäkelä PH, editors. Pneumococcal Vaccines: The Impact of Conjugate Vaccine Washington, DC, USA: ASM Press; 2008. p. 3-18.
112. Grebe T, Hakenbeck R. Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of beta-lactam antibiotics. Antimicrob Agents Chemother 1996;40(4):829-834.
113. Griffith F. The significance of pneumococcal types. J Hyg 1928;27(2):113-159.
114. Guillet M, Zahar JR, Timsit MO, Grandin L, Carbonnelle E, Join-Lambert O, Quesne G, Nassif X, Mejean A, Carbonne A. Horizontal transmission of *Streptococcus pneumoniae* in the surgical ward: a rare source of nosocomial wound infection. Am J Infect Control 2012;40(1):71-72.
115. Gunell M, Hakanen AJ, Aittoniemi J, Kauppila J, Rantakokko-Jalava K, Rissanen A, Saha K, Vaara M, Vuento R, Huovinen P, Nissinen A. Mikrobilääkeresistenssi Suomessa, Finres 1997–2010. FiRe & Terveyden ja hyvinvoinnin laitos (THL) 2012;Raportti 67/2012.
116. Hajdu E, Matuz M, Benko R, Ordas A, Nagy E. An 8-year evaluation of antibiotic consumption and antibiotic resistance among *Streptococcus pneumoniae* from in- and out-patients in Szeged, Hungary. J Chemother 2007;19(5):519-27.
117. Hakenbeck R, Bruckner R, Denapaite D, Maurer P. Molecular mechanisms of beta-lactam resistance in *Streptococcus pneumoniae*. Future Microbiol 2012;7(3):395-410.

118. Hammerschmidt S. Pneumococcal virulence factors and adhesion proteins targeting the host. In: Hakenbeck R, Chhatwal S, editors. Molecular Biology of Streptococci Wymondham, UK: Horizon Biocience; 2007. p. 141-203.
119. Hanage WP. Fuzzy species revisited. BMC Biol 2013;11:41-7007-11-41.
120. Hanage WP, Huang SS, Lipsitch M, Bishop CJ, Godoy D, Pelton SI, Goldstein R, Huot H, Finkelstein JA. Diversity and antibiotic resistance among nonvaccine serotypes of *Streptococcus pneumoniae* carriage isolates in the post-heptavalent conjugate vaccine era. J Infect Dis 2007;195(3):347-352.
121. Hanage WP, Kaijalainen TH, Syrjanen RK, Auranen K, Leinonen M, Makela PH, Spratt BG. Invasiveness of serotypes and clones of *Streptococcus pneumoniae* among children in Finland. Infect Immun 2005;73(1):431-435.
122. Harboe ZB, Benfield TL, Valentiner-Branth P, Hjuler T, Lambertsen L, Kaltoft M, Krogfelt K, Slotved HC, Christensen JJ, Konradsen HB. Temporal trends in invasive pneumococcal disease and pneumococcal serotypes over 7 decades. Clin Infect Dis 2010;50(3):329-337.
123. Hare KM, Smith-Vaughan H, Binks M, Park IH, Nahm MH, Leach AJ. "Dodgy 6As": differentiating pneumococcal serotype 6C from 6A by use of the Quellung reaction. J Clin Microbiol 2009;47(6):1981-1982.
124. Harrison OB, Brueggemann AB, Caugant DA, van der Ende A, Frosch M, Gray S, Heuberger S, Krizova P, Olcen P, Slack M, Taha MK, Maiden MC. Molecular typing methods for outbreak detection and surveillance of invasive disease caused by *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*, a review. Microbiology 2011;157(Pt 8):2181-2195.
125. Hathaway LJ, Brugger SD, Morand B, Bangert M, Rotzetter JU, Hauser C, Graber WA, Gore S, Kadioglu A, Muhlemann K. Capsule type of *Streptococcus pneumoniae* determines growth phenotype. PLoS Pathog 2012;8(3):e1002574.
126. Hausdorff WP, Bryant J, Kloek C, Paradiso PR, Siber GR. The contribution of specific pneumococcal serogroups to different disease manifestations: implications for conjugate vaccine formulation and use, part II. Clin Infect Dis 2000;30(1):122-140.
127. Hausdorff WP, Bryant J, Paradiso PR, Siber GR. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin Infect Dis 2000;30(1):100-121.
128. Hauser C, Aebi S, Muhlemann K. An internationally spread clone of *Streptococcus pneumoniae* evolves from low-level to higher-level penicillin resistance by uptake of penicillin-binding protein gene fragments from nonencapsulated pneumococci. Antimicrob Agents Chemother 2004;48(9):3563-3566.
129. Håvarstein LS. Increasing competence in the genus *Streptococcus*. Mol Microbiol 2010;78(3):541-544.
130. Heidelberger M, Rebers PA. Immunochemistry of the pneumococcal types II. V. and VI. I. The relation of Type VI to Type II and other correlations between chemical constitution and precipitation in antisera to type VI. J Bacteriol 1960;80:145-153.

131. Henrichsen J. Typing of *Streptococcus pneumoniae*: past, present, and future. Am J Med 1999;107(1A):50S-54S.
132. Henrichsen J. Six newly recognized types of *Streptococcus pneumoniae*. J. Clin. Microbiol. 1995;33(10):2759-2762.
133. Henrichsen J, Berntsson E, Kaijser B. Comparison of counterimmunoelectrophoresis and the capsular reaction test for typing of pneumococci. J Clin Microbiol 1980;11(6):589-92.
134. Henriques-Normark B, Tuomanen EI. The pneumococcus: epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med 2013;3(7):10.1101/cshperspect.a010215.
135. Henriques-Normark B, Normark S. Commensal pathogens, with a focus on *Streptococcus pneumoniae*, and interactions with the human host. Exp Cell Res 2010;316(8):1408-1414.
136. Henriques-Normark B, Blomberg C, Dagerhamn J, Battig P, Normark S. The rise and fall of bacterial clones: *Streptococcus pneumoniae*. Nat Rev Microbiol 2008;6(11):827-837.
137. Henriques-Normark B. Molecular epidemiology and mechanisms for antibiotic resistance in *Streptococcus pneumoniae*. In: Hakenbeck R, Chhatwal S, editors. Molecular Biology of Streptococci Wymondham, UK: Horizon Biocience; 2007. p. 269-290.
138. Hicks LA, Harrison LH, Flannery B, Hadler JL, Schaffner W, Craig AS, Jackson D, Thomas A, Beall B, Lynfield R, Reingold A, Farley MM, Whitney CG. Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998-2004. J Infect Dis 2007;196(9):1346-54.
139. Hiller NL, Eutsey RA, Powell E, Earl JP, Janto B, Martin DP, Dawid S, Ahmed A, Longwell MJ, Dahlgren ME, Ezzo S, Tettelin H, Daugherty SC, Mitchell TJ, Hillman TA, Buchinsky FJ, Tomasz A, de Lencastre H, Sa-Leao R, Post JC, Hu FZ, Ehrlich GD. Differences in genotype and virulence among four multidrug-resistant *Streptococcus pneumoniae* isolates belonging to the PMEN1 clone. PLoS One 2011;6(12):e28850.
140. Hiller NL, Janto B, Hogg JS, Boissy R, Yu S, Powell E, Keefe R, Ehrlich NE, Shen K, Hayes J, Barbadora K, Klimke W, Dernovoy D, Tatusova T, Parkhill J, Bentley SD, Post JC, Ehrlich GD, Hu FZ. Comparative genomic analyses of seventeen *Streptococcus pneumoniae* strains: insights into the pneumococcal supragenome. J Bacteriol 2007;189(22):8186-8195.
141. Hollingshead SK, Becker R, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun 2000;68(10):5889-5900.
142. Hoskins J, Alborn WE,Jr, Arnold J, Blaszczak LC, Burgett S, DeHoff BS, Estrem ST, Fritz L, Fu DJ, Fuller W, Geringer C, Gilmour R, Glass JS, Khoja H, Kraft AR, Lagace RE, LeBlanc DJ, Lee LN, Lefkowitz EJ, Lu J, Matsushima P, McAhren SM, McHenney M, McLeaster K, Mundy CW, Nicas TI, Norris FH, O'Gara M, Peery RB, Robertson GT, Rockey P, Sun PM, Winkler ME, Yang Y, Young-Bellido M, Zhao G, Zook CA, Baltz RH,

Jaskunas SR, Rosteck PR,Jr, Skatrud PL, Glass JI. Genome of the bacterium *Streptococcus pneumoniae* strain R6. J Bacteriol 2001;183(19):5709-5717.

143. Hoti F, Erästö P, Leino T, Auranen K. Outbreaks of *Streptococcus pneumoniae* carriage in day care cohorts in Finland - implications for elimination of transmission. BMC Infect Dis 2009;9:102-2334-9-102.
144. Hsieh YC, Lin TL, Chang KY, Huang YC, Chen CJ, Lin TY, Wang JT. Expansion and evolution of *Streptococcus pneumoniae* serotype 19A ST320 clone as compared to its ancestral clone, Taiwan19F-14 (ST236). J Infect Dis 2013;208(2):203-210.
145. Hsu K, Pelton S, Karumuri S, Heisey-Grove D, Klein J, Massachusetts Department of Public Health Epidemiologists. Population-based surveillance for childhood invasive pneumococcal disease in the era of conjugate vaccine. Pediatr Infect Dis J 2005;24(1):17-23.
146. Hsu KK, Shea KM, Stevenson AE, Pelton SI, Massachusetts Department of Public Health. Changing serotypes causing childhood invasive pneumococcal disease: Massachusetts, 2001-2007. Pediatr Infect Dis J 2010;29(4):289-293.
147. Hu FZ, Eutsey R, Ahmed A, Frazao N, Powell E, Hiller NL, Hillman T, Buchinsky FJ, Boissy R, Janto B, Kress-Bennett J, Longwell M, Ezzo S, Post JC, Nesin M, Tomasz A, Ehrlich GD. *In vivo* capsular switch in *Streptococcus pneumoniae* -- analysis by whole genome sequencing. PLoS One 2012;7(11):e47983.
148. Huang SS, Hinrichsen VL, Stevenson AE, Rifas-Shiman SL, Kleinman K, Pelton SI, Lipsitch M, Hanage WP, Lee GM, Finkelstein JA. Continued impact of pneumococcal conjugate vaccine on carriage in young children. Pediatrics 2009;124(1):e1-11.
149. Huang SS, Platt R, Rifas-Shiman SL, Pelton SI, Goldmann D, Finkelstein JA. Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. Pediatrics 2005;116(3):e408-13.
150. Ingels H, Rasmussen J, Andersen PH, Harboe ZB, Glismann S, Konradsen H, Hoffmann S, Valentiner-Branth P, Lambertsen L, Danish Pneumococcal Surveillance Collaboration Group 2009-2010. Impact of pneumococcal vaccination in Denmark during the first 3 years after PCV introduction in the childhood immunization programme. Vaccine 2012;30(26):3944-3950.
151. Ip M, Chau SS, Lai LS, Ma H, Chan PK, Nelson EA. Increased nasopharyngeal carriage of serotypes 6A, 6C, and 6D *Streptococcus pneumoniae* after introduction of childhood pneumococcal vaccination in Hong Kong. Diagn Microbiol Infect Dis 2013;76(2):153-157.
152. Isaacman DJ, McIntosh ED, Reinert RR. Burden of invasive pneumococcal disease and serotype distribution among *Streptococcus pneumoniae* isolates in young children in Europe: impact of the 7-valent pneumococcal conjugate vaccine and considerations for future conjugate vaccines. Int J Infect Dis 2010;14(3):e197-209.
153. Jefferies JM, Clarke SC, Webb JS, Kraaijeveld AR. Risk of Red Queen dynamics in pneumococcal vaccine strategy. Trends Microbiol 2011;19(8):377-81.

154. Jefferies JM, Smith A, Clarke SC, Dowson C, Mitchell TJ. Genetic analysis of diverse disease-causing pneumococci indicates high levels of diversity within serotypes and capsule switching. J Clin Microbiol 2004;42(12):5681-5688.
155. Jenkins SG, Brown SD, Farrell DJ. Trends in antibacterial resistance among *Streptococcus pneumoniae* isolated in the USA: update from PROTEKT US Years 1-4. Ann Clin Microbiol Antimicrob 2008;7:1.
156. Jiang SM, Wang L, Reeves PR. Molecular characterization of *Streptococcus pneumoniae* type 4, 6B, 8, and 18C capsular polysaccharide gene clusters. Infect Immun 2001;69(3):1244-1255.
157. Jin P, Kong F, Xiao M, Oftadeh S, Zhou F, Liu C, Russell F, Gilbert G. First report of putative *Streptococcus pneumoniae* serotype 6D among nasopharyngeal isolates from Fijian children. J Infect Dis 2009;200(9):1375-1380.
158. Johnson HL, Deloria-Knoll M, Levine OS, Stoszek SK, Freimanis Hance L, Reithinger R, Muenz LR, O'Brien KL. Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project. PLoS Med 2010;7(10):10.1371/journal.pmed.1000348.
159. Jourdain S, Dreze PA, Vandeven J, Verhaegen J, Van Melderen L, Smeesters PR. Sequential multiplex PCR assay for determining capsular serotypes of colonizing *S. pneumoniae*. BMC Infect Dis 2011;11:100.
160. Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 2008;6(4):288-301.
161. Kadioglu A, Taylor S, Iannelli F, Pozzi G, Mitchell TJ, Andrew PW. Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. Infect Immun 2002;70(6):2886-2890.
162. Karnezis TT, Smith A, Whittier S, Haddad J, Saiman L. Antimicrobial resistance among isolates causing invasive pneumococcal disease before and after licensure of heptavalent conjugate pneumococcal vaccine. PLoS One 2009;4(6):e5965.
163. Klemets P, Lyytikäinen O, Ruutu P, Kaijalainen T, Leinonen M, Ollgren J, Nuorti J. Trends and geographical variation in invasive pneumococcal infections in Finland. Scand J Infect Dis 2008;40(8):621-8.
164. Ko KS, Baek JY, Song JH. Capsular gene sequences and genotypes of 'serotype 6E' *Streptococcus pneumoniae* isolates. J Clin Microbiol 2013.
165. Ko KS, Baek JY, Song JH. Multidrug-resistant *Streptococcus pneumoniae* serotype 6D clones in South Korea. J Clin Microbiol 2012;50(3):818-822.
166. Koeck JL, Njanpop-Lafourcade BM, Cade S, Varon E, Sangare L, Valjevac S, Vergnaud G, Pourcel C. Evaluation and selection of tandem repeat loci for Streptococcus pneumoniae MLVA strain typing. BMC Microbiol 2005;5:66.
167. Kong F, Brown M, Sabananthan A, Zeng X, Gilbert GL. Multiplex PCR-based reverse line blot hybridization assay to identify 23 *Streptococcus pneumoniae* polysaccharide vaccine serotypes. J Clin Microbiol 2006;44(5):1887-91.

168. Kong F, Wang W, Tao J, Wang L, Wang Q, Sabananthan A, Gilbert GL. A molecular-capsular-type prediction system for 90 *Streptococcus pneumoniae* serotypes using partial *cpsA-cpsB* sequencing and *wzy*- or *wzx*-specific PCR. J Med Microbiol 2005;54(Pt 4):351-6.
169. Kong F, Gilbert GL. Using cpsA-cpsB sequence polymorphisms and serotype-/group-specific PCR to predict 51 *Streptococcus pneumoniae* capsular serotypes. J Med Microbiol 2003;52(Pt 12):1047-1058.
170. Konradsen HB. Validation of serotyping of *Streptococcus pneumoniae* in Europe. Vaccine 2005;23(11):1368-73.
171. Korppi M, Karppa H, Vuento R. Universal pneumococcal vaccination of <2-year-olds decreased pneumococcal bacteremia and antibiotic resistance in <5-year-olds. Acta Paediatr 2013;102(6):e239-40.
172. Kristinsson KG. Epidemiology of penicillin resistant pneumococci in Iceland. Microb Drug Resist 1995;1(2):121-125.
173. Kuch A, Sadowy E, Skoczynska A, Hryniewicz W. First report of *Streptococcus pneumoniae* serotype 6D isolates from invasive infections. Vaccine 2010;28(39):6406-6407.
174. Lahti E, Peltola V, Virkki R, Alanen M, Ruuskanen O. Development of parapneumonic empyema in children. Acta Paediatr 2007;96(11):1686-1692.
175. Lambertsen LM, Harboe ZB, Konradsen HB, Christensen JJ, Hammerum AM. Non-invasive erythromycin-resistant pneumococcal isolates are more often non-susceptible to more antimicrobial agents than invasive isolates. Int J Antimicrob Agents 2010;35(1):72-75.
176. Laupland KB, Gregson DB, Flemons WW, Hawkins D, Ross T, Church DL. Burden of community-onset bloodstream infection: a population-based assessment. Epidemiol Infect 2007;135(6):1037-1042.
177. Lawrence ER, Griffiths DB, Martin SA, George RC, Hall LM. Evaluation of semiautomated multiplex PCR assay for determination of *Streptococcus pneumoniae* serotypes and serogroups. J Clin Microbiol 2003;41(2):601-607.
178. Leimkugel J, Adams Forgor A, Gagneux S, Pfluger V, Flierl C, Awine E, Naegeli M, Dangy JP, Smith T, Hodgson A, Pluschke G. An outbreak of serotype 1 *Streptococcus pneumoniae* meningitis in northern Ghana with features that are characteristic of *Neisseria meningitidis* meningitis epidemics. J Infect Dis 2005;192(2):192-199.
179. Leinonen MK. Detection of pneumococcal capsular polysaccharide antigens by latex agglutination, counterimmunoelectrophoresis, and radioimmunoassay in middle ear exudates in acute otitis media. J Clin Microbiol 1980;11(2):135-40.
180. Leung MH, Bryson K, Freystatter K, Pichon B, Edwards G, Charalambous BM, Gillespie SH. Sequetyping: serotyping *Streptococcus pneumoniae* by a single PCR sequencing strategy. J Clin Microbiol 2012;50(7):2419-2427.
181. Levy C, Varon E, Bingen E, Lecuyer A, Boucherat M, Cohen R, Bacterial Meningitis Study Group. Pneumococcal meningitis in french children before and after the introduction of pneumococcal conjugate vaccine. Pediatr Infect Dis J 2011;30(2):168-170.
182. Lin J, Kaltoft MS, Brandao AP, Echaniz-Aviles G, Brandileone MC, Hollingshead SK, Benjamin WH, Nahm MH. Validation of a multiplex

pneumococcal serotyping assay with clinical samples. J Clin Microbiol 2006;44(2):383-388.
183. Linares J, Ardanuy C, Pallares R, Fenoll A. Changes in antimicrobial resistance, serotypes and genotypes in *Streptococcus pneumoniae* over a 30-year period. Clin Microbiol Infect 2010;16(5):402-410.
184. Lipsitch M, O'Neill K, Cordy D, Bugalter B, Trzcinski K, Thompson CM, Goldstein R, Pelton S, Huot H, Bouchet V, Reid R, Santosham M, O'Brien KL. Strain characteristics of *Streptococcus pneumoniae* carriage and invasive disease isolates during a cluster-randomized clinical trial of the 7-valent pneumococcal conjugate vaccine. J Infect Dis 2007;196(8):1221-7.
185. Liu Z, Nachamkin I, Edelstein PH, Lautenbach E, Metlay JP. Serotype emergence and genotype distribution among macrolide-resistant invasive *Streptococcus pneumoniae* isolates in the postconjugate vaccine (PCV-7) era. Antimicrob Agents Chemother 2012;56(2):743-750.
186. Llull D, Munoz R, Lopez R, Garcia E. A single gene (*tts*) located outside the cap locus directs the formation of *Streptococcus pneumoniae* type 37 capsular polysaccharide. Type 37 pneumococci are natural, genetically binary strains. J Exp Med 1999;190(2):241-251.
187. Loman NJ, Gladstone RA, Constantinidou C, Tocheva AS, Jefferies JM, Faust SN, O'Connor L, Chan J, Pallen MJ, Clarke SC. Clonal expansion within pneumococcal serotype 6C after use of seven-valent vaccine. PLoS One 2013;8(5):e64731.
188. Lund E. Laboratory diagnosis of Pneumococcus infections. Bull World Health Organ 1960;23:5-13.
189. Lynch JP,3rd, Zhanel GG. *Streptococcus pneumoniae:* epidemiology and risk factors, evolution of antimicrobial resistance, and impact of vaccines. Curr Opin Pulm Med 2010;16(3):217-225.
190. Lynch JP,3rd, Zhanel GG. *Streptococcus pneumoniae*: does antimicrobial resistance matter? Semin Respir Crit Care Med 2009;30(2):210-238.
191. Lythgoe KA, Read AF. Catching the Red Queen? The advice of the Rose. Trends Ecol Evol 1998;13(12):473-474.
192. MacLeod CM, Kraus MR. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed in vitro. J Exp Med 1950;92(1):1-9.
193. Malley R, Anderson PW. Serotype-independent pneumococcal experimental vaccines that induce cellular as well as humoral immunity. Proc Natl Acad Sci U S A 2012;109(10):3623-3627.
194. Marchese A, Esposito S, Barbieri R, Bassetti M, Debbia E. Does the adoption of EUCAST susceptibility breakpoints affect the selection of antimicrobials to treat acute community-acquired respiratory tract infections? BMC Infect Dis 2012;12:181.
195. Marks LR, Parameswaran GI, Hakansson AP. Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization *in vitro* and *in vivo*. Infect Immun 2012;80(8):2744-2760.

196. Marks LR, Reddinger RM, Hakansson AP. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in *Streptococcus pneumoniae*. MBio 2012;3(5):e00200-12.
197. Marsh R, Smith-Vaughan H, Hare KM, Binks M, Kong F, Warning J, Gilbert GL, Morris P, Leach AJ. The nonserotypeable pneumococcus: phenotypic dynamics in the era of anticapsular vaccines. J Clin Microbiol 2010;48(3):831-835.
198. Martner A, Skovbjerg S, Paton JC, Wold AE. *Streptococcus pneumoniae* autolysis prevents phagocytosis and production of phagocyte-activating cytokines. Infect Immun 2009;77(9):3826-3837.
199. Massire C, Gertz RE,Jr, Svoboda P, Levert K, Reed MS, Pohl J, Kreft R, Li F, White N, Ranken R, Blyn LB, Ecker DJ, Sampath R, Beall B. Concurrent serotyping and genotyping of pneumococci by use of PCR and electrospray ionization mass spectrometry. J Clin Microbiol 2012;50(6):2018-2025.
200. Mavroidi A, Godoy D, Aanensen DM, Robinson DA, Hollingshead SK, Spratt BG. Evolutionary genetics of the capsular locus of serogroup 6 pneumococci 1. J Bacteriol 2004;186:8181-8192.
201. McCarty M. Discovering genes are made of DNA. Nature 2003;421(6921):406.
202. McEllistrem MC, Nahm MH. Novel pneumococcal serotypes 6C and 6D: anomaly or harbinger. Clin Infect Dis 2012;55(10):1379-1386.
203. McEllistrem MC. Genetic diversity of the pneumococcal capsule: implications for molecular-based serotyping. Future Microbiol 2009;4(7):857-865.
204. McGee L, McDougal L, Zhou J, Spratt BG, Tenover FC, George R, Hakenbeck R, Hryniewicz W, Lefevre JC, Tomasz A, Klugman KP. Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the Pneumococcal Molecular Epidemiology Network. J. Clin. Microbiol. 2001;39(7):2565-2571.
205. Melin M, Trzcinski K, Meri S, Käyhty H, Väkeväinen M. The capsular serotype of *Streptococcus pneumoniae* is more important than the genetic background for resistance to complement. Infect Immun 2010;78(12):5262-5270.
206. Mera RM, Miller LA, Amrine-Madsen H, Sahm DF. The impact of the pneumococcal conjugate vaccine on antimicrobial resistance in the United States since 1996: evidence for a significant rebound by 2007 in many classes of antibiotics. Microb Drug Resist 2009;15(4):261-268.
207. Mera RM, Miller LA, Daniels JJ, Weil JG, White AR. Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States over a 10-year period: Alexander Project. Diagn Microbiol Infect Dis 2005;51(3):195-200.
208. Mercado E, Srinivasan V, Hawkins P, Chochua S, Ochoa T, Beall B, McGee L. First report of *Streptococcus pneumoniae* serotype 6D in South America. J Clin Microbiol 2011;49(5):2080-2081.
209. Messina AF, Katz-Gaynor K, Barton T, Ahmad N, Ghaffar F, Rasko D, H. MG,Jr. Impact of the pneumococcal conjugate vaccine on serotype distribution and antimicrobial resistance of invasive *Streptococcus pneumoniae* isolates in

Dallas, TX, children from 1999 through 2005. Pediatr Infect Dis J 2007;26(6):461-7.

210. Miernyk K, Debyle C, Harker-Jones M, Hummel KB, Hennessy T, Wenger J, Rudolph K. Serotyping of *Streptococcus pneumoniae* isolates from nasopharyngeal samples: use of an algorithm combining microbiologic, serologic, and sequential multiplex PCR techniques. J Clin Microbiol 2011;49(9):3209-14.
211. Millar EV, Pimenta FC, Roundtree A, Jackson D, Carvalho Mda G, Perilla MJ, Reid R, Santosham M, Whitney CG, Beall BW, O'Brien KL. Pre- and post-conjugate vaccine epidemiology of pneumococcal serotype 6C invasive disease and carriage within Navajo and White Mountain Apache communities. Clin Infect Dis 2010;51(11):1258-1265.
212. Miller E, Andrews NJ, Waight PA, Slack MP, George RC. Herd immunity and serotype replacement 4 years after seven-valent pneumococcal conjugate vaccination in England and Wales: an observational cohort study. Lancet Infect Dis 2011;11(10):760-768.
213. Mizrachi Nebenzahl Y, Porat N, Lifshitz S, Novick S, Levi A, Ling E, Liron O, Mordechai S, Sahu RK, Dagan R. Virulence of *Streptococcus pneumoniae* may be determined independently of capsular polysaccharide. FEMS Microbiol Lett 2004;233(1):147-152.
214. MLVA *Streptococcus pneumoniae*. MLVA.eu. Available at: http://www.mlva.eu/pdf/FicheTechnique_v3.pdf. Accessed 15th September, 2013.
215. Moberley S, Holden J, Tatham DP, Andrews RM. Vaccines for preventing pneumococcal infection in adults. Cochrane Database Syst Rev 2013;1:CD000422.
216. Moffitt KL, Malley R. Next generation pneumococcal vaccines. Curr Opin Immunol 2011;23(3):407-413.
217. Montagnani F, Stolzuoli L, Zanchi A, Cresti S, Cellesi C. Antimicrobial susceptibility of *Streptococcus pyogene*s and *Streptococcus pneumoniae*: surveillance from 1993 to 2004 in Central Italy. J Chemother 2006;18(4):389-93.
218. Morais L, Carvalho MdG, Roca A, Flannery B, Mandomando I, Soriano-Gabarro M, Sigauque B, Alonso P, Beall B. Sequential multiplex PCR for identifying pneumococcal capsular serotypes from south-Saharan African clinical isolates. J Med Microbiol 2007;56(9):1181-1184.
219. Morand B, Muhlemann K. Heteroresistance to penicillin in *Streptococcus pneumoniae*. Proc Natl Acad Sci U S A 2007;104(35):14098-14103.
220. Morona JK, Morona R, Paton JC. Comparative genetics of capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* types belonging to serogroup 19. J Bacteriol 1999;181(17):5355-64.
221. Moschioni M, Lo Sapio M, Crisafulli G, Torricelli G, Guidotti S, Muzzi A, Barocchi MA, Donati C. Sequence analysis of 96 genomic regions identifies distinct evolutionary lineages within CC156, the largest *Streptococcus pneumoniae* clonal complex in the MLST database. PLoS One 2013;8(4):e61003.

222. Moschioni M, De Angelis G, Melchiorre S, Masignani V, Leibovitz E, Barocchi MA, Dagan R. Prevalence of pilus-encoding islets among acute otitis media *Streptococcus pneumoniae* isolates from Israel. Clin Microbiol Infect 2010;16(9):1501-1504.
223. Moschioni M, Donati C, Muzzi A, Masignani V, Censini S, Hanage WP, Bishop CJ, Reis JN, Normark S, Henriques-Normark B, Covacci A, Rappuoli R, Barocchi MA. *Streptococcus pneumoniae* contains 3 *rlrA* pilus variants that are clonally related. JID 2008;197.
224. Mudany MA, Kikuchi K, Totsuka K, Uchiyama T. Evaluation of a new serotyping kit for *Streptococcus pneumoniae*. J Med Microbiol 2003;52(Pt 11):975-980.
225. Mueller JE, Yaro S, Ouedraogo MS, Levina N, Njanpop-Lafourcade BM, Tall H, Idohou RS, Sanou O, Kroman SS, Drabo A, Nacro B, Millogo A, van der Linden M, Gessner BD. Pneumococci in the African meningitis belt: meningitis incidence and carriage prevalence in children and adults. PLoS One 2012;7(12):e52464.
226. Mullany P, Allan E, Warburton PJ. Tetracycline resistance genes and mobile genetic elements from the oral metagenome. Clin Microbiol Infect 2012;18 Suppl 4:58-61.
227. Multi locus sequence typing (MLST) of Streptococcus pneumoniae. 2013; Available at: http://spneumoniae.mlst.net/. Accessed 15th September, 2013.
228. Munoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia JJ, Pallares R. Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. Clin Infect Dis 2008;46(2):174-82.
229. Nahm MH, Oliver MB, Siira L, Kaijalainen T, Lambertsen LM, Virolainen A. A report of *Streptococcus pneumoniae* serotype 6D in Europe. J Med Microbiol 2011;60(1):46-48.
230. Nelson AL, Ries J, Bagnoli F, Dahlberg S, Falker S, Rounioja S, Tschop J, Morfeldt E, Ferlenghi I, Hilleringmann M, Holden DW, Rappuoli R, Normark S, Barocchi MA, Henriques-Normark B. RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. Mol Microbiol 2007;66(2):329-340.
231. Nelson ML, Levy SB. The history of the tetracyclines. Ann N Y Acad Sci 2011;1241:17-32.
232. Nesin M, Ramirez M, Tomasz A. Capsular transformation of a multidrug-resistant *Streptococcus pneumoniae* in vivo. J Infect Dis 1998;177(3):707-713.
233. Nunes MC, Madhi SA. Review on the immunogenicity and safety of PCV-13 in infants and toddlers. Expert Rev Vaccines 2011;10(7):951-980.
234. Nunes S, Valente C, Sa-Leao R, de Lencastre H. Temporal trends and molecular epidemiology of recently described serotype 6C of *Streptococcus pneumoniae*. J Clin Microbiol 2009;47:472-474.
235. O'Brien KL, Moisi J, Moulton LH, Madore D, Eick A, Reid R, Weatherholtz R, Millar E, Hu D, Hackell J, Kohberger R, Siber G, Santosham M. Predictors of pneumococcal conjugate vaccine immunogenicity among infants and toddlers in an American Indian PnCRM7 efficacy trial. J Infect Dis 2007;196(1):104-114.

236. Oftadeh S, Satzke C, Gilbert GL. Identification of newly described *Streptococcus pneumoniae* serotype 6D by use of the Quellung reaction and PCR. J. Clin. Microbiol. 2010;48(9):3378-3379.
237. Oliver MB, van der Linden MP, Kuntzel SA, Saad JS, Nahm MH. Discovery of *Streptococcus pneumoniae* serotype 6 variants with glycosyltransferases synthesizing two differing repeating units. J Biol Chem 2013;288(36):25976-25985.
238. Orihuela CJ, Gao G, Francis KP, Yu J, Tuomanen EI. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. J Infect Dis 2004;190(9):1661-1669.
239. Orihuela CJ, Radin JN, Sublett JE, Gao G, Kaushal D, Tuomanen EI. Microarray analysis of pneumococcal gene expression during invasive disease. Infect Immun 2004;72(10):5582-5596.
240. Pai R, Gertz RE, Beall B. Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. J. Clin. Microbiol. 2006;44(1):124-131.
241. Palmu AA, Jokinen J, Borys D, Nieminen H, Ruokokoski E, Siira L, Puumalainen T, Lommel P, Hezareh M, Moreira M, Schuerman L, Kilpi TM. Effectiveness of the ten-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV10) against invasive pneumococcal disease: a cluster randomised trial. Lancet 2013;381(9862):214-222.
242. Park IH, Kim KH, Andrade AL, Briles DE, McDaniel LS, Nahm MH. Nontypeable pneumococci can be divided into multiple *cps* types, including one type expressing the novel gene *pspK*. MBio 2012;3(3):10.1128/mBio.00035.
243. Park IH, Park S, Hollingshead SK, Nahm MH. Genetic basis for the new pneumococcal serotype, 6C. Infect Immun 2007;75:4482-4489.
244. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, Nahm MH. Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. J Clin Microbiol 2007;45:1225-1233.
245. Pebody RG, Morgan O, Choi Y, George R, Hussain M, Andrews N. Use of antibiotics and risk factors for carriage of *Streptococcus pneumoniae*: a longitudinal household study in the United Kingdom. Epidemiol Infect 2009;137(4):555-561.
246. Pebody RG, Hellenbrand W, D'Ancona F, Ruutu P, European Union funded Pnc-EURO contributing group. Pneumococcal disease surveillance in Europe. Euro Surveill 2006;11(9):171-178.
247. Peltola V, Heikkinen T, Ruuskanen O, Jartti T, Hovi T, Kilpi T, Vainionpaa R. Temporal association between rhinovirus circulation in the community and invasive pneumococcal disease in children. Pediatr Infect Dis J 2011;30(6):456-461.
248. Pericone CD, Overweg K, Hermans PW, Weiser JN. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect Immun 2000;68(7):3990-3997.

249. Pichon B, Ladhani SN, Slack MP, Segonds-Pichon A, Andrews NJ, Waight PA, Miller E, George R. Changes in molecular epidemiology of *Streptococcus pneumoniae* causing meningitis following introduction of pneumococcal conjugate vaccination in England and Wales. J Clin Microbiol 2013;51(3):820-827.
250. Pichon B. Pneumococcal MLST oligonucleotide primer sequence, personal communication by email 24th April, 2006.
251. Pihlajamäki M, Jalava J, Huovinen P, Kotilainen P. Antimicrobial resistance of invasive pneumococci in Finland in 1999-2000. Antimicrob Agents Chemother 2003;47(6):1832-5.
252. Pihlajamäki M, Kaijalainen T, Huovinen P, Jalava J. Rapid increase in macrolide resistance among penicillin non-susceptible pneumococci in Finland, 1996-2000. J Antimicrob Chemother 2002;49(5):785-92.
253. Pihlajamäki M, Kataja J, Seppala H, Elliot J, Leinonen M, Huovinen P, Jalava J. Ribosomal mutations in *Streptococcus pneumoniae* clinical isolates. Antimicrob Agents Chemother 2002;46(3):654-8.
254. Pihlajamäki M, Kotilainen P, Kaurila T, Klaukka T, Palva E, Huovinen P, Finnish Study Group for Antimicrobial Resistance (FiRe-Network). Macrolide-resistant *Streptococcus pneumoniae* and use of antimicrobial agents. Clin Infect Dis 2001;33(4):483-488.
255. Pilishvili T, Lexau C, Farley M, Hadler J, Harrison L, Bennett N, Reingold A, Thomas A, Schaffner W, Craig A, Smith P, Beall B, Whitney C, Moore M. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis 2010;201(1):32-41.
256. Pillai D, Shahinas D, Buzina A, Pollock R, Lau R, Khairnar K, Wong A, Farrell D, Green K, McGeer A, Low D. Genome-wide dissection of globally emergent multi-drug resistant serotype 19A *Streptococcus pneumoniae*. BMC Genomics 2009;10(1):642.
257. Pimenta FC, Roundtree A, Soysal A, Bakir M, du Plessis M, Wolter N, von Gottberg A, McGee L, Carvalho Mda G, Beall B. Sequential triplex real-time PCR assay for detecting 21 pneumococcal capsular serotypes that account for a high global disease burden. J Clin Microbiol 2013;51(2):647-652.
258. Pimenta FC, E. GR,Jr, Roundtree A, Yu J, Nahm MH, McDonald RR, Carvalho Mda G, Beall BW. Rarely occurring 19A-like *cps* locus from a serotype 19F pneumococcal isolate indicates continued need of serology-based quality control for PCR-based serotype determinations. J Clin Microbiol 2009;47(7):2353-4.
259. Platt S, Pichon B, George R, Green J. A bioinformatics pipeline for high-throughput microbial multilocus sequence typing (MLST) analyses. Clin Microbiol Infect 2006;12(11):1144-6.
260. Pletz MW, Maus U, Krug N, Welte T, Lode H. Pneumococcal vaccines: mechanism of action, impact on epidemiology and adaption of the species. Int J Antimicrob Agents 2008;32(3):199-206.
261. Pneumococcal Molecular Epidemiology Network (PMEN). Available at: http://www.sph.emory.edu/PMEN/. Accessed 11th September, 2013.

262. Porat N, Benisty R, Trefler R, Ozalvo D, Givon-Lavi N, Dagan R. Baseline epidemiology and genetic structure of *Streptococcus pneumoniae* serotype 6D in southern Israel prior to introduction of pneumococcal conjugate vaccines. J Clin Microbiol 2013;51(5):1580-1582.
263. Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys JP. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science 2006;313(5783):89-92.
264. Rakov AV, Ubukata K, Robinson DA. Population structure of hyperinvasive serotype 12F, clonal complex 218 *Streptococcus pneumoniae* revealed by multilocus boxB sequence typing. Infect Genet Evol 2011;11(8):1929-1939.
265. Rantala M. Antimicrobial resistance in *Streptococcus pneumoniae* in Finland with special reference to macrolides and telithromycin. PhD Thesis, University of Helsinki. 2009.
266. Rantala M, Huikko S, Huovinen P, Jalava J. Prevalence and molecular genetics of macrolide resistance among *Streptococcus pneumoniae* isolates collected in Finland in 2002. Antimicrob Agents Chemother 2005;49(10):4180-4.
267. Regev-Yochay G, Hanage WP, Trzcinski K, Rifas-Shiman SL, Lee G, Bessolo A, Huang SS, Pelton SI, McAdam AJ, Finkelstein JA, Lipsitch M, Malley R. Re-emergence of the type 1 pilus among *Streptococcus pneumoniae* isolates in Massachusetts, USA. Vaccine 2010;28(30):4842-4846.
268. Riedel S, Beekmann SE, Heilmann KP, Richter SS, Garcia-de-Lomas J, Ferech M, Goosens H, Doern GV. Antimicrobial use in Europe and antimicrobial resistance in *Streptococcus pneumoniae*. Eur J Clin Microbiol Infect Dis 2007;26(7):485-90.
269. Rinta-Kokko H, Jokinen J, Siira L, Palmu AA, Virtanen MJ, Nohynek H, Virolainen A, Nuorti JP. Impact of 10-valent pneumococcal conjugate vaccine (PCV10) against invasive pneumococcal disease (IPD) among vaccine-eligible children in Finland. 31st Meeting of the European Society for Paediatric Infectious Diseases (ESPID), Milan, Italy, 28 May - 1 June, 2013 2013.
270. Roberts J, Chandra M, Pebody R, Stuart J. Variation in incidence of pneumococcal and meningococcal disease across Europe. Euro Surveill 2007;12(11):E071115.5.
271. Ruckinger S, van der Linden M, Reinert RR, von Kries R, Burckhardt F, Siedler A. Reduction in the incidence of invasive pneumococcal disease after general vaccination with 7-valent pneumococcal conjugate vaccine in Germany. Vaccine 2009;27(31):4136-4141.
272. Sabat AJ, Budimir A, Nashev D, Sa-Leao R, van Dijl J, Laurent F, Grundmann H, Friedrich AW, ESCMID Study Group of Epidemiological Markers (ESGEM). Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro Surveill 2013;18(4):20380.
273. Sadowy E, Kuch A, Gniadkowski M, Hryniewicz W. Expansion and evolution of the *Streptococcus pneumoniae* Spain9V-ST156 clonal complex in Poland. Antimicrob. Agents Chemother. 2010;54(5):1720-1727.
274. Sadowy E, Izdebski R, Skoczynska A, Grzesiowski P, Gniadkowski M, Hryniewicz W. Phenotypic and molecular analysis of penicillin-nonsusceptible

Streptococcus pneumoniae isolates in Poland. Antimicrob. Agents Chemother. 2007;51(1):40-47.

275. Salter SJ, Hinds J, Gould KA, Lambertsen L, Hanage WP, Antonio M, Turner P, Hermans PW, Bootsma HJ, O'Brien KL, Bentley SD. Variation at the capsule locus, *cps*, of mistyped and non-typable *Streptococcus pneumoniae* isolates. Microbiology 2012;158(Pt 6):1560-1569.
276. Satzke C, Ortika BD, Oftadeh S, Russell FM, Robins-Browne RM, Mulholland EK, Gilbert GL. Molecular epidemiology of *Streptococcus pneumoniae* serogroup 6 isolates from Fijian children, including newly identified serotypes 6C and 6D. J Clin Microbiol 2010;48(11):4298-300.
277. Scott JA, Hall AJ, Dagan R, Dixon JM, Eykyn SJ, Fenoll A, Hortal M, Jette LP, Jorgensen JH, Lamothe F, Latorre C, Macfarlane JT, Shlaes DM, Smart LE, Taunay A. Serogroup-specific epidemiology of *Streptococcus pneumoniae*: associations with age, sex, and geography in 7,000 episodes of invasive disease. Clin Infect Dis 1996;22(6):973-981.
278. Scott JR, Hinds J, Gould KA, Millar EV, Reid R, Santosham M, O'Brien KL, Hanage WP. Nontypeable pneumococcal isolates among navajo and white mountain apache communities: are these really a cause of invasive disease? J Infect Dis 2012;206(1):73-80.
279. Selva L, Ciruela P, Blanchette K, del Amo E, Pallares R, Orihuela CJ, Munoz-Almagro C. Prevalence and clonal distribution of pcpA, psrP and Pilus-1 among pediatric isolates of *Streptococcus pneumoniae*. PLoS One 2012;7(7):e41587.
280. Selva L, del Amo E, Brotons P, Munoz-Almagro C. Rapid and easy identification of capsular serotypes of *Streptococcus pneumoniae* by use of fragment analysis by automated fluorescence-based capillary electrophoresis. J Clin Microbiol 2012;50(11):3451-3457.
281. Shin J, Baek JY, Kim SH, Song JH, Ko KS. Predominance of ST320 among *Streptococcus pneumoniae* serotype 19A isolates from 10 Asian countries. J Antimicrob Chemother 2011;66(5):1001-1004.
282. Simoes AS, Pereira L, Nunes S, Brito-Avo A, de Lencastre H, Sa-Leao R. Clonal evolution leading to maintenance of antibiotic resistance rates among colonizing pneumococci in the PCV7 era in Portugal. J Clin Microbiol 2011;49(8):2810-2817.
283. Singleton R, Wenger J, Klejka JA, Bulkow LR, Thompson A, Sarkozy D, Emini EA, Gruber WC, Scott DA. The 13-valent pneumococcal conjugate vaccine for invasive pneumococcal disease in Alaska native children: results of a clinical trial. Pediatr Infect Dis J 2013;32(3):257-263.
284. Sjöström K, Blomberg C, Fernebro J, Dagerhamn J, Morfeldt E, Barocchi MA, Browall S, Moschioni M, Andersson M, Henriques F, Albiger B, Rappuoli R, Normark S, Henriques-Normark B. Clonal success of piliated penicillin nonsusceptible pneumococci. Proc Natl Acad Sci U S A 2007;104:12907-12912.
285. Sjöström K, Spindler C, Ortqvist A, Kalin M, Sandgren A, Kühlmann-Berenzon S, Henriques-Normark B. Clonal and capsular types decide whether

pneumococci will act as a primary or opportunistic pathogen. Clinical Infectious Diseases 2006;42(4):451-459.
286. Skogberg K, Lyytikäinen O, Ollgren J, Nuorti JP, Ruutu P. Population-based burden of bloodstream infections in Finland. Clin Microbiol Infect 2012;18(6):E170-6.
287. Skogberg K, Lyytikäinen O, Ruutu P, Ollgren J, Nuorti JP. Increase in bloodstream infections in Finland, 1995-2002. Epidemiol Infect 2008;136(1):108-114.
288. Skov Sorensen UB, Blom J, Birch-Andersen A, Henrichsen J. Ultrastructural localization of capsules, cell wall polysaccharide, cell wall proteins, and F antigen in pneumococci. Infect Immun 1988;56(8):1890-1896.
289. Smith AM, Klugman KP. Alterations in PBP 1A essential-for high-level penicillin resistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 1998;42(6):1329-1333.
290. Sogstad MKR, Hoiby EA, Caugant DA. Molecular characterization of non-penicillin-susceptible *Streptococcus pneumoniae* in Norway. J. Clin. Microbiol. 2006;44(9):3225-3230.
291. Song JH, Dagan R, Klugman KP, Fritzell B. The relationship between pneumococcal serotypes and antibiotic resistance. Vaccine 2012;30(17):2728-2737.
292. Song JH, Baek JY, Ko KS. Comparison of capsular genes of *Streptococcus pneumoniae* serotype 6A, 6B, 6C, and 6D isolates. J Clin Microbiol 2011;49(5):1758-1764.
293. Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ. Displaying the relatedness among isolates of bacterial species -- the eBURST approach. FEMS Microbiol Lett 2004;241:129-134.
294. Spratt BG, Greenwood BM. Prevention of pneumococcal disease by vaccination: does serotype replacement matter? Lancet 2000;356(9237):1210-1211.
295. Steenhoff AP, Shah SS, Ratner AJ, Patil SM, McGowan KL. Emergence of vaccine-related pneumococcal serotypes as a cause of bacteremia. Clin Infect Dis 2006;42(7):907-14.
296. Steinman RM, Moberg CL. A triple tribute to the experiment that transformed biology. J Exp Med 1994;179(2):379-384.
297. Syrjänen RK, Kilpi TM, Kaijalainen TH, Herva EE, Takala AK. Nasopharyngeal carriage of *Streptococcus pneumoniae* in Finnish children younger than 2 years old. J Infect Dis 2001;184(4):451-459.
298. Talbot TR, Poehling KA, Hartert TV, Arbogast PG, Halasa NB, Mitchel E, Schaffner W, Craig AS, Edwards KM, Griffin MR. Reduction in high rates of antibiotic-nonsusceptible invasive pneumococcal disease in Tennessee after introduction of the pneumococcal conjugate vaccine. Clin Infect Dis 2004;39(5):641-8.
299. Rokottajan käsikirja, penumokokkirokotukset. Terveyden ja hyvinvoinnin laitos. Available at: http://www.thl.fi/fi_FI/web/rokottajankasikirja-fi/pneumokokkirokotukset. Accessed 12th September, 2013.

300. THL Statistical Database. Terveyden ja hyvinvoinnin laitos. Available at: http://www3.thl.fi/stat/. Accessed 21st September, 2013.
301. Tettelin H, Nelson KE, Paulsen IT, Eisen JA, Read TD, Peterson S, Heidelberg J, DeBoy RT, Haft DH, Dodson RJ, Durkin AS, Gwinn M, Kolonay JF, Nelson WC, Peterson JD, Umayam LA, White O, Salzberg SL, Lewis MR, Radune D, Holtzapple E, Khouri H, Wolf AM, Utterback TR, Hansen CL, McDonald LA, Feldblyum TV, Angiuoli S, Dickinson T, Hickey EK, Holt IE, Loftus BJ, Yang F, Smith HO, Venter JC, Dougherty BA, Morrison DA, Hollingshead SK, Fraser CM. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science 2001;293(5529):498-506.
302. GenBank. The National Center for Biotechnology Information (NCBI). Available at: http://www.ncbi.nlm.nih.gov/genbank. Accessed 14th September, 2013.
303. Basic Local Alignment Search Tool (BLAST). The National Center for Biotechnology Information (NCBI). Available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed 17th August, 2012.
304. Thomas JC, Pettigrew MM. Multilocus Sequence Typing and Pulsed Field Gel Electrophoresis of Otitis Media Causing Pathogens. In: Sokolowski B, editor. Auditory and Vestibular Research: Methods and Protocols; 2008.
305. Tilley SJ, Orlova EV, Gilbert RJ, Andrew PW, Saibil HR. Structural basis of pore formation by the bacterial toxin pneumolysin. Cell 2005;121(2):247-256.
306. Tomita Y, Okamoto A, Yamada K, Yagi T, Hasegawa Y, Ohta M. A new microarray system to detect *Streptococcus pneumoniae* serotypes. J Biomed Biotechnol 2011;2011:352736.
307. Tuomanen E, Liu H, Hengstler B, Zak O, Tomasz A. The induction of meningeal inflammation by components of the pneumococcal cell wall. J Infect Dis 1985;151(5):859-868.
308. Vainio A, Kaijalainen T, Hakanen AJ, Virolainen A. Prevalence of pilus-encoding islets and clonality of pneumococcal isolates from children with acute otitis media. Eur J Clin Microbiol Infect Dis 2011;30(4):515-519.
309. Vainio A, Lyytikäinen O, Sihvonen R, Kaijalainen T, Teirilä L, Rantala M, Lehtinen P, Ruuska P, Virolainen A. An outbreak of pneumonia associated with *S. pneumoniae* at a military training facility in Finland in 2006. APMIS 2009;117(7):488-91.
310. Valles X, Marcos A, Pinart M, Piner R, Marco F, Mensa JM, Torres A. Hospitalized community-acquired pneumonia due to *Streptococcus pneumoniae*: Has resistance to antibiotics decreased? Chest 2006;130(3):800-806.
311. van Cuyck H, Pichon B, Leroy P, Granger-Farbos A, Underwood A, Soullie B, Koeck JL. Multiple-locus variable-number tandem-repeat analysis of *Streptococcus pneumoniae* and comparison with multiple loci sequence typing. BMC Microbiol 2012;12:241-2180-12-241.
312. van de Sande-Bruinsma N, Grundmann H, Verloo D, Tiemersma E, Monen J, Goossens H, Ferech M, European Antimicrobial Resistance Surveillance System Group, European Surveillance of Antimicrobial Consumption Project

Group. Antimicrobial drug use and resistance in Europe. Emerg Infect Dis 2008;14(11):1722-1730.
313. van der Linden M, Winkel N, Kuntzel S, Farkas A, Perniciaro SR, Reinert RR, Imohl M. Epidemiology of *Streptococcus pneumoniae* serogroup 6 isolates from IPD in children and adults in Germany. PLoS One 2013;8(4):e60848.
314. van der Linden M, Al-Lahham A, Nicklas W, Reinert RR. Molecular characterization of pneumococcal isolates from pets and laboratory animals. PLoS One 2009;4(12):e8286.
315. van Hoek AJ, Andrews N, Waight PA, George R, Miller E. Effect of serotype on focus and mortality of invasive pneumococcal disease: coverage of different vaccines and insight into non-vaccine serotypes. PLoS One 2012;7(7):e39150.
316. van Selm S, van Cann LM, Kolkman MA, van der Zeijst BA, van Putten JP. Genetic basis for the structural difference between *Streptococcus pneumoniae* serotype 15B and 15C capsular polysaccharides. Infect Immun 2003;71(11):6192-6198.
317. Vanderkooi OG, Low DE, Green K, Powis JE, McGeer A, Toronto Invasive Bacterial Disease Network. Predicting antimicrobial resistance in invasive pneumococcal infections. Clin Infect Dis 2005;40(9):1288-1297.
318. Varaldo PE, Montanari MP, Giovanetti E. Genetic elements responsible for erythromycin resistance in streptococci. Antimicrob Agents Chemother 2009;53(2):343-353.
319. Vaz M, Meirinhos-Soares L, Sousa CC, Ramirez M, Melo-Cristino J, Lopes JA. Serotype discrimination of encapsulated *Streptococcus pneumoniae* strains by Fourier-transform infrared spectroscopy and chemometrics. J Microbiol Methods 2013;93(2):102-107.
320. Vestrheim DF, Steinbakk M, Aaberge IS, Caugant DA. Postvaccination increase in serotype 19A pneumococcal disease in Norway is driven by expansion of penicillin-susceptible strains of the ST199 complex. Clin Vaccine Immunol 2012;19(3):443-445.
321. Vollmer W. Structure and biosynthesis of the pneumococcal cell wall. In: Hakenbeck R, Chhatwal S, editors. Molecular Biology of Streptococci Wymondham, UK: Horizon Biocience; 2007. p. 83-117.
322. von Gottberg A, Cohen C, de Gouveia L, Meiring S, Quan V, Whitelaw A, Crowther-Gibson P, Madhi SA, Whitney CG, Klugman KP. Epidemiology of invasive pneumococcal disease in the pre-conjugate vaccine era: South Africa, 2003-2008. Vaccine 2013;31(38):4200-4208.
323. Waite RD, Penfold DW, Struthers JK, Dowson CG. Spontaneous sequence duplications within capsule genes *cap8E* and *tts* control phase variation in *Streptococcus pneumoniae* serotypes 8 and 37. Microbiology 2003;149(Pt 2):497-504.
324. Waite RD, Struthers JK, Dowson CG. Spontaneous sequence duplication within an open reading frame of the pneumococcal type 3 capsule locus causes high-frequency phase variation. Mol Microbiol 2001;42(5):1223-1232.
325. Watson DA, Musher DM, Jacobson JW, Verhoef J. A brief history of the pneumococcus in biomedical research: a panoply of scientific discovery. Clin Infect Dis 1993;17(5):913-924.

326. Wei H, Håvarstein LS. Fratricide is essential for efficient gene transfer between pneumococci in biofilms. Appl Environ Microbiol 2012;78(16):5897-5905.
327. Weinberger DM, Malley R, Lipsitch M. Serotype replacement in disease after pneumococcal vaccination. Lancet 2011;378(9807):1962-1973.
328. Weinberger DM, Harboe ZB, Sanders EA, Ndiritu M, Klugman KP, Ruckinger S, Dagan R, Adegbola R, Cutts F, Johnson HL, O'Brien KL, Scott JA, Lipsitch M. Association of serotype with risk of death due to pneumococcal pneumonia: a meta-analysis. Clin Infect Dis 2010;51(6):692-699.
329. Weinberger DM, Trzcinski K, Lu YJ, Bogaert D, Brandes A, Galagan J, Anderson PW, Malley R, Lipsitch M. Pneumococcal capsular polysaccharide structure predicts serotype prevalence. PLoS Pathog 2009;5(6):e1000476.
330. Weinstein MP, Klugman KP, Jones RN. Rationale for revised penicillin susceptibility breakpoints versus *Streptococcus pneumoniae*: coping with antimicrobial susceptibility in an era of resistance. Clin Infect Dis 2009;48(11):1596-1600.
331. Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, Lynfield R, Reingold A, Cieslak PR, Pilishvili T, Jackson D, Facklam RR, Jorgensen JH, Schuchat A, Active Bacterial Core Surveillance of the Emerging Infections Program Network. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N Engl J Med 2003;348(18):1737-1746.
332. WHO Publication. Pneumococcal vaccines WHO position paper - 2012 - recommendations. Vaccine 2012;30(32):4717-4718.
333. WHO Publication. Pneumococcal conjugate vaccine for childhood immunization--WHO position paper. Wkly Epidemiol Rec 2007;82(12):93-104.
334. Wierzbowski AK, Karlowsky JA, Adam HJ, Nichol KA, Hoban DJ, Zhanel GG, on behalf of the Canadian Antimicrobial Resistance Alliance (CARA). Evolution and molecular characterization of macrolide-resistant *Streptococcus pneumoniae* in Canada between 1998 and 2008. J Antimicrob Chemother 2013.
335. Wierzbowski AK, Nichol K, Laing N, Hisanaga T, Nikulin A, Karlowsky JA, Hoban DJ, Zhanel GG. Macrolide resistance mechanisms among *Streptococcus pneumoniae* isolated over 6 years of Canadian Respiratory Organism Susceptibility Study (CROSS) (1998 2004). J Antimicrob Chemother 2007;60(4):733-40.
336. Wyres KL, Lambertsen LM, Croucher NJ, McGee L, von Gottberg A, Linares J, Jacobs MR, Kristinsson KG, Beall BW, Klugman KP, Parkhill J, Hakenbeck R, Bentley SD, Brueggemann AB. Pneumococcal capsular switching: a historical perspective. J Infect Dis 2013;207(3):439-449.
337. Wyres KL, Lambertsen LM, Croucher NJ, McGee L, von Gottberg A, Linares J, Jacobs MR, Kristinsson KG, Beall BW, Klugman KP, Parkhill J, Hakenbeck R, Bentley SD, Brueggemann AB. The multidrug-resistant PMEN1 pneumococcus is a paradigm for genetic success. Genome Biol 2012;13(11):R103.

338. Xu X, Cai L, Xiao M, Kong F, Oftadeh S, Zhou F, Gilbert GL. Distribution of serotypes, genotypes, and resistance determinants among macrolide-resistant *Streptococcus pneumoniae* isolates. Antimicrob. Agents Chemother. 2010;54(3):1152-1159.
339. Yildirim I, Hanage WP, Lipsitch M, Shea KM, Stevenson A, Finkelstein J, Huang SS, Lee GM, Kleinman K, Pelton SI. Serotype specific invasive capacity and persistent reduction in invasive pneumococcal disease. Vaccine 2010;29(2):283-288.
340. Yother J. Capsules of *Streptococcus pneumoniae* and other bacteria: paradigms for polysaccharide biosynthesis and regulation. Annu Rev Microbiol 2011;65:563-581.
341. Yu J, Lin J, Kim KH, H. BW,Jr, Nahm MH. Development of an automated and multiplexed serotyping assay for *Streptococcus pneumoniae*. Clin Vaccine Immunol 2011;18(11):1900-7.
342. Yu J, Lin J, Benjamin WH,Jr, Waites KB, Lee CH, Nahm MH. Rapid multiplex assay for serotyping pneumococci with monoclonal and polyclonal antibodies. J Clin Microbiol 2005;43(1):156-162.
343. Zähner D, Gudlavalleti A, Stephens DS. Increase in pilus islet 2-encoded pili among *Streptococcus pneumoniae* isolates, Atlanta, Georgia, USA. Emerg Infect Dis 2010;16(6):955-62.
344. Zapun A, Contreras-Martel C, Vernet T. Penicillin-binding proteins and beta-lactam resistance. FEMS Microbiol Rev 2008;32(2):361-385.
345. Zartler ER, Porambo RJ, Anderson CL, Chen LH, Yu J, Nahm MH. Structure of the capsular polysaccharide of pneumococcal serotype 11A reveals a novel acetylglycerol that is the structural basis for 11A subtypes. J Biol Chem 2009;284(11):7318-7329.
346. Zhuo F, Xiao M, Kong F, Oftadeh S, Zhou F, Zhang J, Gilbert GL. Prevalence and genetic diversity of pneumococcal serogroup 6 in Australia. Clin Microbiol Infect 2011;17(8):1246-1253.
347. Zulz T, Wenger JD, Rudolph K, Robinson DA, Rakov AV, Bruden D, Singleton RJ, Bruce MG, Hennessy TW. Molecular characterization of *Streptococcus pneumoniae* serotype 12F isolates associated with rural community outbreaks in Alaska. J Clin Microbiol 2013;51(5):1402-1407.